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13. ABSTRACT (Maximum 200 Words)

MDM2 is a feedback regulator of p53 and is central to apoptotic response of prostate cancer cells to radiotherapy (RT), androgen deprivation (AD) and RT+AD. Our in vitro measurements demonstrate that antisense-MDM2 (AS-MDM2) significantly enhances apoptosis in response to all of these treatments. The increase in apoptosis translates into an increase in overall cell death, determined using clonogenic assay. An orthotopic model using LNCaP cells injected into the prostates of nude mice corroborates the in vitro findings, particularly in terms of sensitization to AD. The mouse model involves determinations of growth inhibition through measurements of serum PSA and micro-MRI-based tumor volume. Treatment with AS-MDM2+AD and AS-MDM2+AD+RT resulted in the greatest growth inhibition, compared to the other groups. All prostate cancer risk groups stand to benefit.

We have also measured MDM2 expression using immunohistochemistry in men treated on Radiation Therapy Oncology group trials 86-10 and 92-02. MDM2 overexpression is associated with a higher rate of distant metastasis and mortality, independent of conventional factors, treatment, ki-67 and p53. We now have a method not only for identifying men at high risk of treatment failure, but also for selecting men who would have the greatest potential benefit from therapeutically targeting MDM2.

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INTRODUCTION

Androgen deprivation (AD) continues to be one of the most common treatments for prostate cancer; yet, the mechanisms of action are poorly understood. Normal prostate epithelial cells are induced into apoptosis in over 80% of cells within 10 days of AD. In contrast, the apoptotic response of prostate tumor cells tumors occurs in the minority, with the majority induced into a resting state. In order to take full advantage of AD as a therapeutic modality, the mechanisms responsible for the diminished apoptotic response of tumor cells must be understood. Our data indicate that MDM2 has a central role in this process; antisense-MDM2 (AS-MDM2) restores the apoptotic response of prostate cancer cells to AD.

Prostate cancer responds to radiotherapy (RT), which is an important treatment modality for men without distant metastasis. In those with high risk features, local relapse is relatively common. The apoptotic response of prostate cancer cells to radiation is minimal and most cell death appears to be mitotic. Our data show that MDM2 increases the apoptotic and overall cell death responses of prostate cancer cells to RT. In addition, we demonstrate that the expression of MDM2 in men treated with RT±AD is a determinant of outcome.

The scope of this research is that through manipulating MDM2 expression via antisense-MDM2 (AS-MDM2) it may be possible to improve the efficacy of RT, AD and the combination. Virtually every patient, from those with early disease to those with distant metastasis, stands to benefit.

BODY

Task 1. Determine the impact of MDM2 suppression and overexpression on the interaction of AD and RT in promoting cell death and inhibiting prostate cancer growth in vitro and in vivo.

a. Complete in vitro apoptosis measurements on LNCaP cells treated in vitro with AS in combination with AD, RT, and AD+RT. Months 1-6.
b. Baseline cell viability, cell number apoptosis, and clonogenic assays of LNCaP-MST. Months 1-6.
c. Time course experiments of AS effects on AD, RT, and AD+RT in LNCaP and LNCaP-MST cells. Months 7-12.
d. In vivo experiments of the action of AS on LNCaP and LNCaP-MST cells. Months 7-36

Table 1. Freedom From Biochemical and MRI-Based Tumor Volume Failure in LNCaP Cells Grown Orthotopically in the Prostates of Nude Mice

Group	FFBF	FFTVF	Combined
No Tx	0%(0/8)	0%(0/8)	0%(0/8)
MM	0%(0/8)	0%(0/8)	0%(0/8)
AS-MDM2	11%(1/9)	25%(2/8)	13%(1/8)
AD	55%(5/9)	33%(3/9)	22%(2/9)
MM+RT	0%(0/5)	20%(1/5)	0%(0/5)
AS+RT	0%(0/10)	20%(2/10)	0%(0/10)
AS+AD	67%(6/9)	67%(6/9)	67%(6/9)
AS+AD+RT	90%(1/10)	70%(7/10)	70%(7/10)

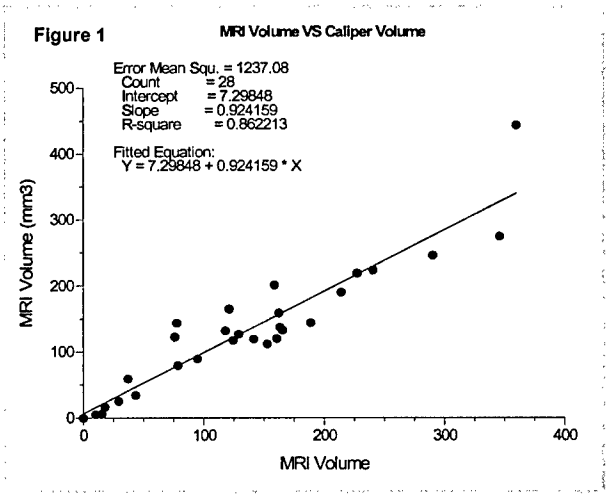
No Tx= no treatment; MM= mismatch control at 25 mg/kg injected intraperitoneally for 5 days/week for 3 weeks; AD= androgen deprivation via orchiectomy started 3 days prior to AS-MDM2; AS= AS-MDM2 at 25 mg/kg injected intraperitoneally for 5 days/week for 3 weeks; RT= 5 Gy pelvic radiation therapy given after 5 days of AS treatment; FFBF= freedom from a PSA of >1.5 ng/mL at 6 weeks from treatment start; FFTVF= freedom from a MRI-based tumor volume of >10 mm^3 at 10 weeks from treatment.

The MM+AD and MM+AD+RT groups are not completed yet.

We have completed components a-c in Task 1 and the results have been presented at two meetings (1, 2) and published in two papers (3, 4). Since the last annual report, we have focused on the in vivo studies (Section d of Task 1). The studies using LNCaP cells are nearly complete (Table 1) and those with LNCaP-MST are ongoing.

Table 1 summarizes the preliminary results of LNCaP cells grown orthotopically in the prostates of nude mice. We are currently treating with AS-

MDM2 (25 mg/kg per injection) for 15 days and administering 5 Gy RT as a single fraction on day 5, in the middle of the AS-MDM2 injections. AD is initiated 3 days prior to the start of the AS-MDM2.



The in vivo experiments are thus far reflective of the in vitro results. **Table 1** displays the findings of freedom from biochemical failure (FFBF) modeled after Cowen et al (5) from our group. Biochemical failure is defined as a PSA >1.5 ng/mL at 6 weeks after treatment start. Tumor volume is also being quantified using micro-MR imaging. MRI-based tumor volume failure is defined as a volume of >10 cc at 10 weeks after treatment start. Intraperitoneal injections of AS-MDM2 sensitized LNCaP cells grown orthotopically in the prostates of nude mice to AD and AD + RT. It is too early to tell if there will be a significant difference between AS-MDM2+AD and AS-MDM2+AD+RT; but, there is a improvement in freedom from failure in these groups relative to the others. Preliminary analysis of the PSA and MRI-based tumor volume doubling times for the tumors that have evidence of growth after treatment also indicates that the tumors treated with AS-MDM2+AD+RT are growing slower than those treated with AS-MDM2+AD. These data extend our observations that when tumors recur after temporary AD+RT, they grow at a slower rate as compared to RT or AD alone(6, 7). AS-MDM2 when combined with short term AD+RT appears to promote even greater slowing of tumor growth when failure is evident. The results with AS-MDM2 in vivo are striking and suggest that this strategy will be of particular benefit to men with high risk apparently clinically localized disease, who are commonly treated with AD+RT, as well as those with documented metastatic disease, who are usually treated with AD alone.

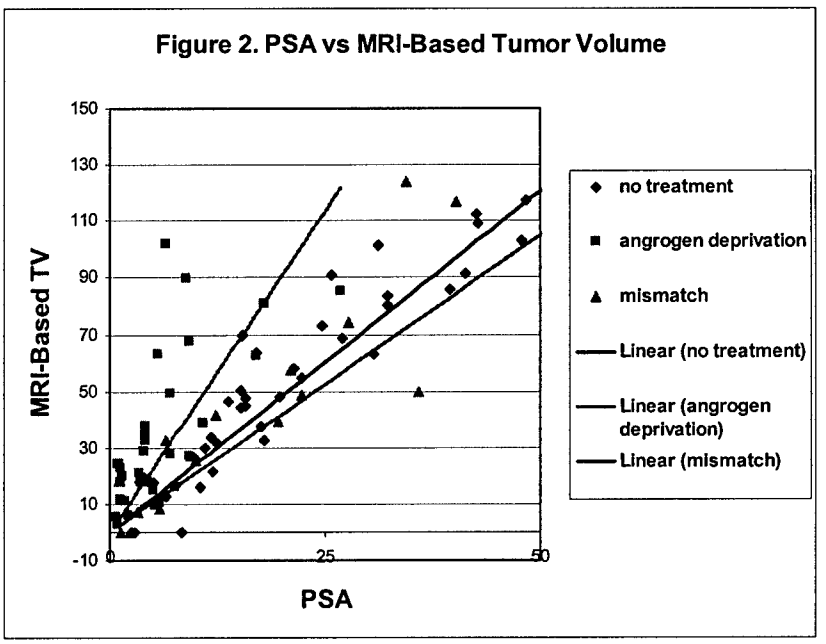


Figure 3. E2F-1 Expression After Ad-E2F-1

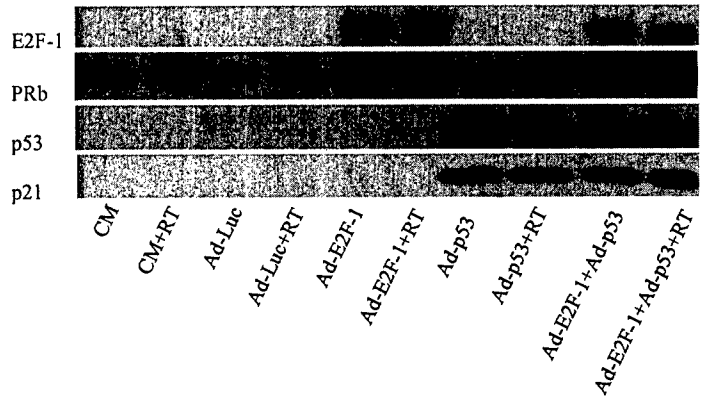
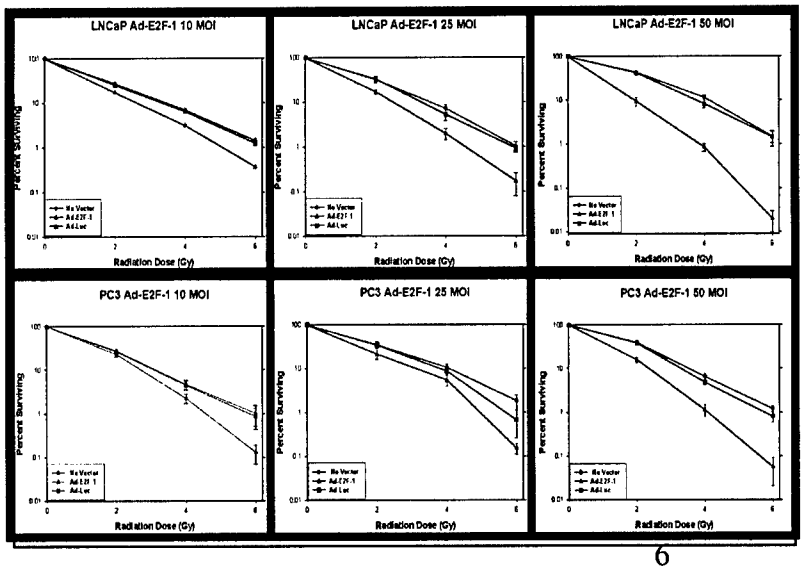


Figure 1 shows the association between MRI-based tumor volume and the volume of resected tumors measured with calipers. The caliper measurements correlated strongly (linear regression R^2 = 0.80) with the MRI-based measurements, as well as with tumor volume measurements. Another unique aspect of this dataset is the ability to determine the relationship between PSA and MRI-based tumor volume under different conditions. **Figure 2** demonstrates that under the conditions of AD there is a change in this relationship such that lower levels of PSA correspond to higher MRI-based tumor volumes. To our knowledge, these are the first data to show that the ratio of PSA to tumor volume is lower after AD. In summary, the micro-MRI imaging technique we have developed has allowed for 1) more accurate in vivo determination of the timing of relapse, 2) the documentation of the relationships between serum PSA and tumor volume and 3) a comparison of PSA and tumor volume kinetics under the various treatments being tested.

Task 2. Define the molecular mechanisms underlying the changes in LNCaP cell killing in response to AD ± RT when MDM2 is suppressed or overexpressed.

a. Western blot analysis of p53, p21, MDM2, bcl-2, bax, E2F-1 and pRB under conditions of AD and AS given simultaneously. Months 1-12.
b. Western blot analysis of p53, p21, MDM2, bcl-2, bax, E2F-1 and pRB under conditions of AD given 2 d before AS. Months 13-24.
c. Manipulation of gene expression to further enhance/replace the action of AD, RT, or AD+RT based on the Western results from the studies in years 1 and 2; for example, targeting p53 using adenoviral-p53, E2f-1 using adenoviral E2F-1, or bcl-2 using antisense bcl-2. Months 25-36

Figure 4. Clonogenic survival after Ad-E2F + RT



Most of the proposed LNCaP Western blot analyses were included in the two papers that were described above (3, 4). These analyses included the protein levels of MDM2, p53, and p21. AS-MDM2 caused a reduction in MDM2, which was even further reduced by AD. P53 and p21 increased after AS-MDM2 or RT. We have also looked at bcl-2 and bax levels in response to AS-MDM2, and have not found much of an effect. More recently, we have initiated the measurement of mRNA expression using the Oligo

GE Array (SuperArray Bioscience Corp, Frederick, MD). A number of genes have been found to be increased over two fold with AS-MDM2 over the mismatch control; for example, bax was elevated to a greater degree than bcl-2. These experiments are in progress.

As per Task 2c, we have been using adenoviral-E2F-1 (Ad-E2F-1) to sensitize cells to radiation. **Figure 3** displays the overexpression E2F-1 and p53 from exogenously adding Ad-E2F-1 and/or Ad-p53, respectively in $p53^{null}$ PC3 cells. Both $p53^{wild\text{-}type}$ LNCaP and $p53^{null}$ PC3 cells express E2F-1 (this is not evident in Figure 3 because of the conditions used). Our results show that Ad-E2F-1 is a potent sensitizer of LNCaP and PC3 cells. Ad-E2F-1 increases the apoptotic response and reduces clonogenicity (**Figure 4**) of prostate cancer cells to RT. When Ad-p53 is added with E2F-1 to PC3 cells, there appeared to be an additive increase in clonogenic cell death, demonstrating that, although the response of prostate cancer cells to Ad-E2F-1 is not completely dependent on p53, the presence of p53 promotes increased cell killing with RT. An article containing these findings has been favorably reviewed by the International Journal of Radiation Oncology Biology and Physics. These data have led us to postulate that, like MDM2, E2F-1 will affect an enhanced cell death response to AD ± RT as well. Since MDM2 and E2F-1 are intimately associated in the mediating apoptosis, we have included the further investigation of the interaction of these apoptotic regulatory proteins as an Aim in a new Department of Defense grant recently submitted.

Task 3. Examine the degree and predictive value of MDM2 overexpression in diagnostic archival tissue specimens from patients treated with RT alone and RT + AD.

a. MDM2 immunohistochemistry analysis of 110 cases from RTOG protocol 86-10. Months 1-6.
b. Statistical analysis of MDM2 staining results from RTOG protocol 86-10. Months 7-10.
c. MDM2 immunohistochemistry analysis of cases from RTOG protocol 92-02. Months 7-30.
d. Statistical analysis of MDM2 staining results from RTOG protocol 92-02. Months 30-36.

The immunohistochemical staining and analysis of MDM2 expression in 109 diagnostic samples from patients treated in RTOG 86-10 has been completed. The work initially was presented at last years American Society of Therapeutic Radiation Oncologists annual meeting (8), and a paper on this work (See Appendix) has been accepted for publication in Cancer (9). The nuclear staining of MDM2 expression was quantified manually and using an image analysis system (ACIS, ChromaVision, San Juan Capistrano, CA). The strongest relationship to outcome was found for the ACIS determination of the percentage of cells staining positive (PSP) positive. Using the ACIS index, MDM2 overexpression was a borderline significant predictor of distant metastasis in univariate and multivariate analysis ($p=0.06$).

Recently we completed the analysis of MDM2 expression in cases treated in RTOG 92-02, which has been accepted for presentation at the 2005 meeting of the American Radium Society (Barcelona, Spain). A different antibody for immunohistochemical staining, which resulted in improved nuclear staining relative to background, was used. There were 1514 analyzable cases in the parent cohort of RTOG 92-02 (short term AD plus RT versus long term AD plus RT) and, of these, MDM2 expression analysis was possible in 586 (38.7%). The analysis was similar to that used for RTOG protocol 86-10 in that image analysis was used to quantify the PSP. The per sample mean intensity score (MIS, relative units) was also measured.

No difference in length of follow-up (median 71 months), biochemical failure (BF, ASTRO definition), local failure (LF), distant metastasis (DM), cause specific mortality (CSM) or overall mortality (OM) was seen between the parent and MDM2 cohorts. The median PSP was 40.5% (range 0-100) and the median MIS was 167 (range 0-228). In univariate analysis, PSP was not associated with any of the endpoints tested, as a continuous or dichotomous variable (median cutpoint). As a continuous variable, MIS was significantly related to BF (p=0.01) and weakly related to CSM (p=0.08) and OM (p=0.08). As a dichotomous variable (median cutpoint), MIS was significantly related to BF (p=0.02), DM (p=0.0074) and CSM (p=0.04), and weakly associated with OM (p=0.08). In multivariate analysis using Cox proportional hazards regression, dichotomized MIS was significantly independent of age, Gleason score, PSA, stage and assigned treated in predicting for BF (p=0.01), DM (p=0.02) and CSM (p=0.04), and was weakly associated with OM (p=0.06). The results support using MDM2 expression in patient stratification and a rationale for targeting MDM2 using an antisense approach.

Another biomarker that has been reported to be predictive of outcome in the same patient cohorts is p53 (10). The overexpression of p53, using more than 20% of the cells with nuclear staining as the criterion for overexpression, was related significantly to distant metastasis. Since MDM2 feedback regulates p53, an analysis was performed to determine if MDM2 overexpresion is independent and perhaps complementary to p53 in terms of distant metastasis risk. Ki-67 was also included in this analysis, as we have previously established that Ki-67 has a pronounced association with distant metastasis in men treated with RT ± AD (11-13).

There were 384 cases in RTOG 92-02 (25.4%) with adequate tissue for immunohistochemical analysis of all three biomarkers (Ki-67, MDM2 and p53). Median follow-up was 96.3 mo. Image analysis was used to quantify the percentage of tumor nuclei staining positive (PSP) and per sample mean intensity score (MIS) for Ki-67 and MDM2. For p53, the PSP was based on manual counts - image analysis was no better. Cox proportional hazards multivariate analysis (MVA) was used to determine the independence of the biomarkers for local failure (LF), biochemical failure (BF), distant metastasis (DM) and overall mortality (OM). The biomarkers were tested as continuous and dichotomized (Ki-67 $\leq$9% vs >9% [n=135]; p53 <20% vs $\geq$20% [n=92]; MDM2 PSP $\leq$40.5% vs >40.5% [n=219, median cutpoint] and MIS $\leq$167 vs >167 [n=204, median cutpoint]) variables based on prior analyses. Events were measured from randomization. The other covariates included were dichotomized in accordance with RTOG 92-02 stratification and randomization criteria as follows: median Age ($\leq$70, >70 years), pretreatment PSA ($\leq$30, >30 ng/mL), T-stage (T2c, T3-T4), Gleason score (2-6, 7-10), and assigned treatment (Short Term AD+RT, Long Term AD+RT). There were no statistically significant differences in outcome for those with all biomarker data, versus those with missing data, by any of the endpoints tested. Although all of the covariates mentioned were tested in each MVA, only the results for Ki-67 and MDM2 are shown (Table). p53 was significant only in univariate analysis. As a continuous variable in MVA MDM2 PSP was significantly associated with BF, while MDM2 MIS predicted for BF, DM and OM. As dichotomous variables MDM2 PSP predicted for BF and MDM2 MIS for OM. A composite MDM2 dichotomous covariate of those with PSP >40.5% and MIS >167 (n=159) versus the others, was significantly related to BF, DM and OM. In univariate analysis, the MDM2 composite endpoint of overexpression was associated with a doubling of DM from 10 to 20% and a nearly 10% reduction in survival. Ki-67 was significantly related in MVA to LF, BF and DM when used as a continuous or dichotomous variable. MDM2 overexpression is a robust determinant of BF, DM and OM that is independent of traditional prognostic factors, treatment, p53, and Ki-67, and is a promising therapeutic target.

KEY RESEARCH ACCOMPLISHMENTS

- AS-MDM2 sensitizes LNCaP prostate cancer cells to androgen deprivation, radiation and the combination. This is the first study to demonstrate such an interaction.
- Apoptosis appears to be the major cell death pathway affected by AS-MDM2.
- MDM2 overexpressing LNCaP-MST cells were more resistant to the sensitizing action of AS-MDM2, confirming the role of MDM2 in the development of prostate cancer cell resistance to androgen deprivation.
- AS-MDM2 increases p53 and p21 expression, demonstrating that the negative feedback pathway was intact and that p53
- MDM2 is common in tumors from men with locally advanced prostate cancer and is associated with an increased risk of distant metastasis. These are the first studies to examine the relationship of MDM2 expression to patient outcome after radiotherapy with or without androgen deprivation.
- MDM2 overexpression is predictive of distant metastasis and mortality independent of conventional factors, treatment, p53 and Ki-67.

REPORTABLE OUTCOMES

1. **Pollack A,** Mu Z, Hachem P, Agrawal S. "Modulation of Prostate Cancer Cell Death in Response to Androgen Deprivation and Radiation by MDM2. International Conference on Translational Research, Lugano, Switzerland 3/17/03
2. **Pollack A**, Hanlon AL, Movsas B, Hanks GE, Uzzo R, Horwitz EM. Biochemical failure as a determinant of distant metastasis and death in prostate cancer treated with brachytherapy. Int J Radiat Oncol Biol Phys 57:19-23, 2003.
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CONCLUSIONS

The studies described indicate that MDM2 plays an important role in the response of prostate cancer cells to RT, AD and RT+AD. The in vitro studies in LNCaP and LNCaP-MST cell lines illustrate the link between manipulating apoptosis and increasing overall cell killing by clonogenic assay. The in vivo studies are in progress, but have been supportive of the in vitro experiments, although the relative benefit of AS-MDM2+AD+RT over AS-MDM2+AD has not been shown and is underpowered. The cost of trying to resolve this difference is prohibitive. Taken together, the in vitro and in vivo data indicate that AS-MDM2 holds promise as a therapeutic strategy for nearly every risk group with prostate cancer. Those with localized favorable to intermediate risk disease may benefit from the use of lower doses of RT and consequently reduced side effects. Those with localized high risk disease are usually treated with AD+RT and have a significant risk of microscopic nodal and distant metastasis. The potentiation of the response of prostate cancer cells to AD by AS-MDM2 makes this approach particularly attractive. More recent data (not shown) indicate that AS-MDM2 even has activity in cells that demonstrate no growth inhibition to AD and in bcl-2-overexpressing LNCaP cells that display less than expected growth rate inhibition to AD.

The analysis of MDM2 expression by immunohistochemistry in archival tissue from RTOG protocols 86-10 and 92-02 reflect the preclinical antisense studies. MDM2 overexpression is associated with an increased risk of distant metastasis and death, which is independent of whether the patients received RT alone, RT + short term AD or RT + long term AD. Moreover, the significance of MDM2 overexpression was also independent of p53 and Ki-67. MDM2 expression is turning out to be one of the most important determinants of outcome yet investigated. We now have a method not only for identifying men at high risk of treatment failure, but also for selecting men who would have the greatest potential benefit from therapeutically targeting MDM2.

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APPENDICES

Mu Z, Hachem P, Agrawal S, *et al.* Antisense MDM2 sensitizes prostate cancer cells to androgen deprivation, radiation, and the combination. *Int J Radiat Oncol Biol Phys* 2004;58:336-343.

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ICTR 2003 **Translational Research and Pre-Clinical Strategy Study**

ANTISENSE MDM2 SENSITIZES PROSTATE CANCER CELLS TO ANDROGEN DEPRIVATION, RADIATION, AND THE COMBINATION

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Purpose: Antisense MDM2 (AS) sensitizes a variety of tumor cell types, including prostate cancer, to radiation and chemotherapy. We have previously described that AS enhances the apoptotic response to androgen deprivation (AD) and that this translates into a reduction in overall cell survival, as measured by clonogenic assay. Because AD + radiation (RT) is a key strategy for the treatment of men with high-risk prostate cancer, AS was tested for the ability to sensitize cells to the combination of AD+RT.

Methods and Materials: LNCaP cells were cultured *in vitro* in either complete, androgen deprived (AD), or AD+R1881 (synthetic androgen) medium for 2–3 days before AS was administered. Radiation at 5 Gy was given 18–24 h later. Processing of the cells after RT was done at 3 h for Western blots, 24 and 48 h for trypan blue dye exclusion, 18 h for Annexin V staining by flow cytometric analysis, 18 h for Caspase 3+7 quantification by fluorometric assay, and immediately for clonogenic survival measured 12–14 days later. There were 18 treatment groups that were studied: lipofectin control, AS, antisense mismatch (ASM), AD, AD+R1881, and RT in all possible combinations. Statistical comparisons between groups were accomplished with one-way analysis of variance using the Bonferroni test, considering all 18 groups.

Results: AS caused a reduction in MDM2 expression and an increase in p53 and p21 expression. Early cell death by trypan blue was found to be reflective of the apoptotic results by Annexin V and Caspase 3+7. AS caused a significant increase in apoptosis over the lipofectin control, AD, and RT controls. Apoptosis was further increased significantly by the addition of AD or RT to AS. When AS, AD, and RT were combined, there was a consistent increase in early cell death over AS+AD and AS+RT by all of the assay methods, although this increase was not significant. Overall cell death measured by clonogenic assay revealed synergistic cell killing of AS+RT beyond that of ASM+RT and RT alone, and AS+RT+AD beyond that of AS+RT, AS+RT+AD+R1881, ASM+RT+AD, and ASM+RT+AD+R1881.

Conclusion: AS sensitizes cells to AD, RT, and AD+RT and shows promise in the treatment of the full range of patients with prostate cancer. AS has the potential to sensitize the primary tumor to AD+RT and metastasis to AD. © 2004 Elsevier Inc.

Antisense, MDM2, Androgen deprivation, Radiation, Prostate cancer.

INTRODUCTION

The combination of androgen deprivation (AD) plus radiation (RT) has become the standard for patients with high-risk prostate cancer. Despite the documentation of a survival improvement from this combination over RT alone in some series (1–3), there are still questions regarding the long-term efficacy over AD alone (4). An understanding of the molecular events that occur in the response of cells to AD and RT could lead to novel strategies that enhance cell killing in response to these agents, thereby allowing for the potential to reduce toxicity through reduced exposure. It may be possible even to replace AD and RT altogether with less morbid alternative biologic therapies. Our approach has been to manipulate the apoptotic pathway.

Recently, we focused on MDM2 as a target for enhancing the apoptotic response of LNCaP cells to AD. The rationale was that MDM2 is overexpressed in 30–40% of prostate cancers (5, 6), MDM2 regulates p53 expression through a negative feedback loop (7), and p53 has been implicated in the apoptotic response of prostate epithelial cells to AD (8–12). An effective method for ablating MDM2 expression

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is through antisense MDM2 (AS) (13–16). Prior studies from our group have shown that AS+AD results in increased apoptosis over that seen by AS, AD, antisense mismatch (ASM), or ASM+AD (17). The pattern of increased early apoptotic cell death was mirrored in clonogenic survival assays, suggesting that overall cell death of LNCaP cells was significantly enhanced by the addition of AS to AD. Because AS has been shown to sensitize cells to RT and chemotherapy in a number of cell lines, it was hypothesized that AS will sensitize prostate cancer cells not only to AD and RT given individually, but also to AD+RT. Wild-type p53–expressing human LNCaP cells were chosen for the investigation of the effects of AS on AD+RT.

METHODS AND MATERIALS

Antisense oligonucleotides

The oligonucleotides were provided by Hybridon, Inc. (Cambridge, MA). The antisense MDM2 oligonucleotide (AS) and its mismatch control oligonucleotide (ASM) are 20-mer mixed-backbone oligonucleotides with following sequence (AS; 5′-UGACACCTGTTCTCACUCAC-3′) and (ASM; 5′-UGTCACCCTTTTTCATUCAC-3′). They were stored as frozen aliquots at −20°C.

Cell culture system

LNCaP cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium–F12 medium, containing 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin (complete medium [CM]), as described previously (18). Cells were typically cultured in complete medium before the culture conditions were altered. Androgen deprivation was achieved by culturing the cells in medium containing 10% charcoal-stripped serum (AD medium). Androgen was replaced by adding the synthetic androgen R1881 (NEN Life Science Products, Boston, MA) at 1×10^{-10} M to AD medium (18).

Western blot analyses

Protein levels of MDM2, p53, p21, Bcl-2, Bax, E2F1, pRb, and β-actin were analyzed after different treatments. Cells were cultured in complete, AD, or AD+R1881 medium for 3 days and incubated with 200 nM of AS or ASM in 4 mL culture medium for 24 h in the presence of 7 μg/mL lipofectin (Invitrogen, Carlsbad, CA). Three hours after γ-irradiation to 5 Gy (RT) using a ^{137}Cs irradiator (Model 81-14R, J.L. Shepherd & Associates, San Fernando, CA), cells were lysed in a lysis buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate [SDS] with protease inhibitor cocktail set I [Calbiochem, San Diego, CA]) and were sonicated for 30 s on ice. Protein concentration was determined using the BCA protein assay reagent kit (Pierce, Rockford, IL). Identical amounts of protein were fractionated by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were then incubated in blocking buffer (phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat milk) for 1 h at room temperature and were washed twice with the washing buffer (phosphate-buffered saline containing 0.1% Tween 20) for 5 min. The membranes were then incubated with the appropriate primary antibody: anti-MDM2 monoclonal antibody (mAb) at 1:1000; anti-p53 mAb at 1:1000; anti-p21 mAb at 1:1000; anti-Rb mAb at 1:1000, anti-β actin at 1:5000 dilution or anti-E2F1 mAb at 1:1000 dilution (all antibodies from Calbiochem, San Diego, CA), anti-Bcl-2 mAb at 1:1000 (DAKO A/S, Carpinteria, CA), or anti-Bax polyclonal IgG at 1:1000 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. Membranes were washed and then incubated with 1:2000 diluted sheep anti-mouse IgG or donkey-rabbit IgG horseradish peroxidase–conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at room temperature. After the washes were repeated, the proteins of interest were detected by the enhanced chemiluminescence reagents according to the manufacturer's directions (Amersham, Aylesbury, UK).

Trypan blue cell viability assay

Early overall cell viability was assessed by trypan blue dye exclusion. Cells were seeded at 5×10^4 cells/well in 24-well plates and cultured in complete, AD, or AD+R1881 medium for 2–3 days. Cells were then transfected with 200 nM of AS or ASM in the presence of lipofectin (7 μg/mL). After 24 h, cells were irradiated to 5 Gy. The percentage of dead cells was measured by trypan blue dye exclusion at 24 and 48 h after treatment; typical cumulative cell death rates after AS treatment were 37% and 52%. From these data, the 48-h time point was chosen to be representative.

Measurements of apoptosis

Apoptosis was confirmed by Annexin V staining and Caspase 3+7 activity assays. LNCaP cells (2×10^5) were cultured in complete, AD, or AD+R1881 medium for 2–3 days. Cells were then incubated with 200 nM AS or ASM in the presence of lipofectin (7 μg/mL) for 18 h. Cells were then irradiated to 5 Gy. After 24 h, all cells (floating and attached) were harvested by trypsinization and labeled with Annexin V–PE and 7-amino-actinomycin D (7-AAD) (Guava Technologies Inc., Burlingame, CA) according to the manufacturer's instructions and analyzed by flow cytometry on a GuavaPC personal flow cytometer (Guava Technologies Inc., Burlingame, CA).

Caspase 3+7 activity was measured using a fluorometric substrate, Z-DEVD-Rhodamine (The Apo-ONE Homogeneous Caspase-3/7 Assay kit; Promega, Madison, WI). Cells were cultured for 2–3 days in CM, AD medium, or AD+R1881 medium and then incubated with AS or ASM for 18 h. Different times for AS exposure and the delay in performing the assay after RT were tested, and 18-h times were found to be representative, without excessive activity. Cells were then irradiated to 5 Gy. After 18 h, a total of 5×10^4 cells in 100 μL culture medium were mixed with 100 μL of Homogeneous Caspase-3/7 reagent in 96-well plates and incubated at room temperature for 18 h. Substrate cleavage was

Table 1. Western blot analyses of the effects of AD and/or RT on densitometry measurements of the expression of key proteins in the apoptotic pathway

Group	n*	CM+RT÷ CM	AD+RT÷ AD	AD+R1881+RT÷ AD+R1881
p53	5	6.8 ± 2.0	2.8 ± 0.6	4.5 ± 1.6
p21	3	3.0 ± 0.3	9.5 ± 2.5	7.7 ± 1.1
Bcl-2	5	0.9 ± 0.1	1.1 ± 0.1	0.6 ± 0.1
Bax	3	0.5 ± 0.2	0.7 ± 0.1	1.1 ± 0.1
MDM2	4	11.8 ± 3.3	26.8 ± 7.5	13.9 ± 5.2
E2F1	3	1.1 ± 0.2	1.9 ± 0.3	1.9 ± 0.8
pRb	2	0.9 ± 0.1	0.9 ± 0.2	0.8 ± 0.3

* n = number of Western blot analyses done.

Note: The relative changes in band density measured by densitometry are shown as mean ± SEM.

quantified fluorometrically at 485-nm excitation and 538-nm emission. Fluorescence was measured on a fluorescent plate reader (LabSystems Inc., Franklin, MA). For a control, caspase 3+7 activity was inhibited by adding Ac-DEVD-CHO (Promega, Madison, WI) to the cell culture before the assay.

Radiation treatment and clonogenic assay

Cells were cultured in complete, AD, or AD+R1881 medium for 2–3 days and then incubated with 200 nM AS or ASM in the presence of lipofectin (7 μg/mL). After 24 h, cells were irradiated to 2, 4, and 6 Gy. Immediately after irradiation, cells were trypsinized and serially diluted, and known numbers of cells were replated into 100-mm dishes. The plates were incubated for 12–14 days and stained with 0.25% methylene blue. The colonies were counted using an automated counter (Imaging Products International, Inc., Chantilly, VA). The clonogenic survival results were corrected for differences in plating efficiency from the various culture conditions. The dilutions for clonogenic assay were done in triplicate, and the results were averaged together (intraexperimental averages). The data shown in the clonogenic survival table represent the average from multiple experiments (interexperimental average).

RESULTS

Western blot analyses

MDM2 was identified as a potential target to enhance the response of prostate cancer cells to AD and RT through an investigation of the changes induced by these conditions in the expression of a variety of proteins involved in the apoptotic pathway. Table 1 displays the changes of MDM2, p53, p21, bcl-2, bax, E2F1, and pRb protein levels to AD ± RT, as determined by densitometry measurements of the resultant bands from Western blot analyses. The ratios of the band densities are shown. The average of 4 experiments of MDM2 revealed an 11.8-fold and a 26.8-fold increase in expression of MDM2 for CM+RT over CM and AD+RT over AD alone, respectively. When R1881 was added, the ratio of AD+RT over AD alone fell back to nearly the level of the CM+RT over CM ratio. The changing level of MDM2 in response AD and RT was reflective of the changes in apoptosis under these conditions (18, 19). For these reasons, combined with the findings that p53 influences the apoptotic response of prostate epithelial cells to AD (8, 9), MDM2 was targeted using an antisense strategy.

Figure 1 displays representative Western blots showing that AS almost completely abrogated radiation-induced MDM2 expression in either complete, AD, or AD+R1881 medium, whereas ASM had little effect. The level of p53 increased after AS or RT treatment; ASM also increased the level of p53, as well as p21, but to a lesser degree. The mechanism for the slight increase in p53 levels after exposure to ASM is unclear, although in other Western blots, MDM2 seemed to be elevated from ASM treatment. The level of p21 was not increased by RT treatment, but was increased by AS treatment. AD alone had little effect on the protein levels of MDM2, p53, or p21. The expression of MDM2 seemed to be slightly higher for AS+AD+RT as compared to AS+AD, AS+RT, and AS+AD+R1881+RT. There was no obvious change in bcl-2 or bax expression by Western blot analysis in response to AS, AD, or RT (not shown).

Early cell death after AS ± AD ± RT treatment

The ability of AS to enhance the response of LNCaP cells to AD and/or RT was first evaluated using trypan blue dye exclusion. The cells were exposed to 200 nM AS, with or without AD, for 24 h, followed by γ-irradiation (5 Gy). A summary of three experiments measuring cell death 48 h after radiation is shown in Table 2. Eighteen treatment groups were analyzed together using analysis of variance. The statistics for the group comparisons are shown relative to the group above. Additional comparisons showed that AS resulted in significantly less cell death than AS+AD or AS+RT; these latter groups had about the same level of cell death. When R1881 was added to AS+AD, there was a reduction in cell death back to the level of AS. When AS was added to AD+RT, cell death was enhanced over all of the other groups, but the differences beyond that seen with AS+AD and AS+RT were not significant.

Direct measurements of apoptosis were performed to determine the contribution of apoptosis to early overall cell death that was quantified above by trypan blue staining. Apoptosis was measured directly by Annexin V binding. Cells were cultured in either complete, AD, or AD+R1881 medium for 48 h and then incubated with 200 nM AS or ASM for 18 h, followed by γ-irradiation (5 Gy). Twenty-four hours after irradiation, cells were prepared for Annexin V–PE and 7-AAD staining. Table 3 shows that early apoptosis (Annexin V–PE-positive and 7-AAD–negative) was higher from AS+AD (36.6% apoptosis) and AS+RT (32.7%) treatments over either AS (22.2%), AD (6.7%), or RT (3.9%) treatments given individually. These findings were significant (Table 2). However, there was no significant difference between AS+AD or AS+RT and AD+AS+RT, although the level of apoptosis was consistently higher in the AD+AS+RT group.

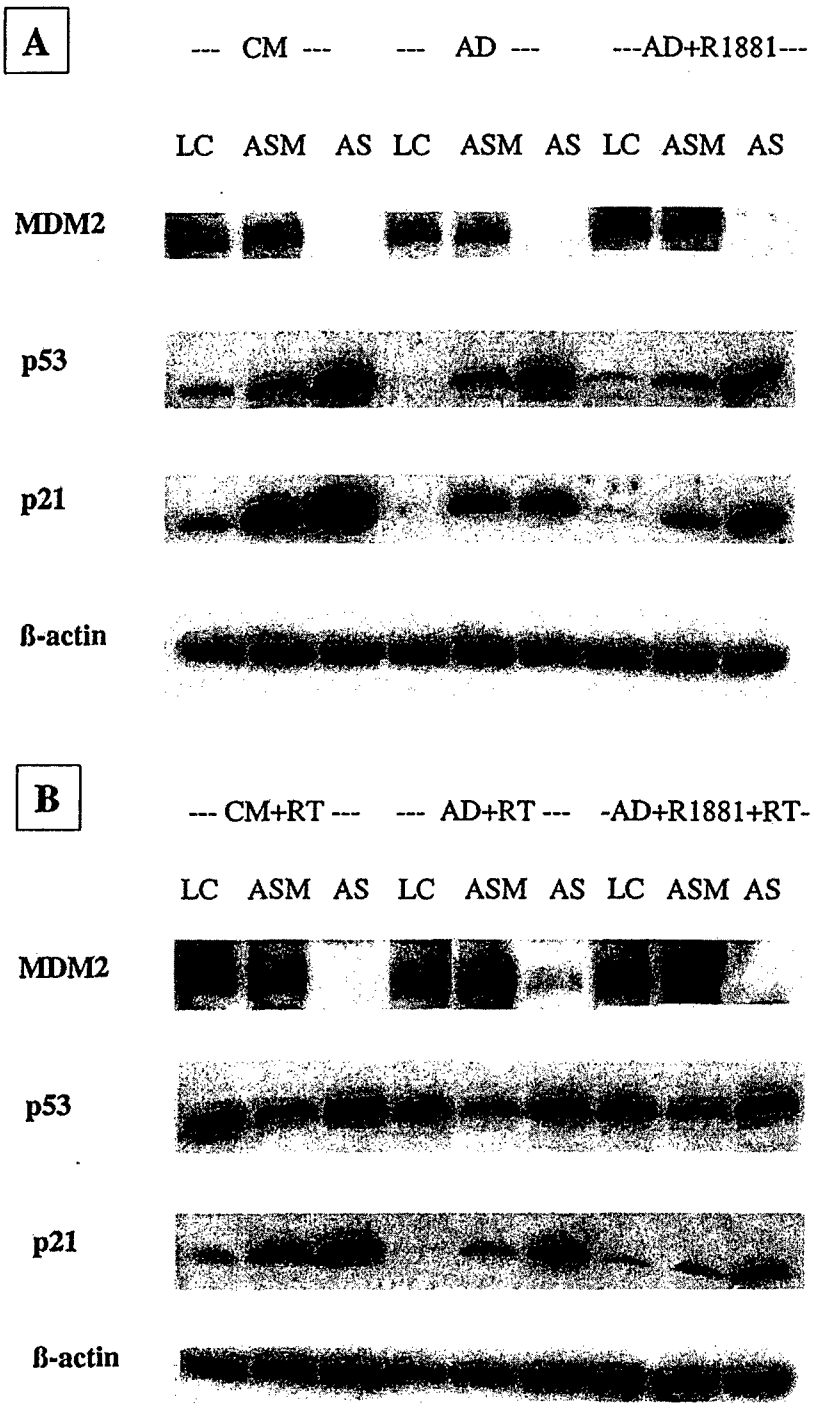

Fig. 1. Western blot analyses of LNCaP cells grown for 2–3 days in CM, AD, or AD+R1881 medium. AS or ASM was administered at 200 nM; 24 h later, RT at 5 Gy was given. The cells were harvested 3 h later, and the protein was extracted for analysis of MDM2, p53, p21, and β-actin levels. (A) Without RT; (B) With RT.

The pattern of apoptotic cell death observed by the Annexin V assay was very similar to that from the Caspase 3+7 assay. As shown in Table 4, Caspase 3+7 activity was increased from AS+AD or AS+RT as compared to AS, AD or RT treatments given singly. There was no significant increase in apoptosis from AS+AD+RT over that from AS+AD or AS+RT. Caspase 3+7 activity was inhibited by the addition of R1881 to AS+AD to approximately the levels of AS alone. Moreover, the addition of specific caspase inhibitor Ac-DEVD-CHO (data not shown) reduced caspase 3+7 activity. These results suggest that AS accentuates LNCaP tumor cell apoptosis to AD and RT through p53 by activating caspase 3+7.

Table 2. Trypan blue quantification of early cell death

Treatment	Mean	SEM	p*
Lipofectin control	10.8	0.5	–
AS†	52.0	3.4	<0.0001
ASM	24.8	1.4	<0.0001
AD	21.3	1.4	1.000
AD+AS†,‡	71.0	3.9	<0.0001
AD+ASM	31.8	1.9	<0.0001
AD+R1881	15.5	2.1	0.226
AD+AS+R1881†	57.0	5.6	<0.0001
AD+ASM+R1881	30.0	3.9	<0.0001
Lipofectin control+RT	21.8	2.2	1.000
AS+RT‡§	69.5	3.4	<0.0001
ASM+RT	30.8	3.7	<0.0001
AD+RT‡	26.3	2.2	1.000
AD+AS+RT§	82.3	3.5	<0.0001
AD+ASM+RT	39.3	4.2	<0.0001
AD+R1881+RT	19.3	1.1	0.02
AD+R1881+AS+RT§	69.0	4.1	<0.0001
AD+R1881+ASM+RT	35.8	6.7	<0.0001

Abbreviations: AS = antisense MDM2; ASM = antisense mismatch; AD = androgen deprivation; RT = radiation therapy; SEM = standard error of the mean.

* Compared to group above, one-way ANOVA, Bonferroni test. The average of 4 experiments is shown.

† AD+AS vs. AS (p = 0.039); AD+AS vs. AD+AS+R1881 (p = 0.855).

‡ AD+AS vs. AS+RT (p = 1.000); AD+AS vs. AD+RT (p < 0.0001).

§ AD+AS vs. AD+AS+RT (p = 1.000); AS+RT vs. AD+AS+RT (p = 1.000); AD+AS+RT vs. AD+R1881+AS+RT (p = 1.000).

Table 3. Annexin V quantification of early apoptosis

Treatment	Mean	SEM	p*
Lipofectin control	3.4	0.7	–
AS†	22.2	0.5	<0.0001
ASM	7.4	0.4	<0.0001
AD	6.7	0.8	1.000
AD+AS†‡	36.6	1.2	<0.0001
AD+ASM	12.1	1.1	<0.0001
AD+R1881	5.7	1.1	0.225
AD+AS+R1881†	28.6	2.0	<0.0001
AD+ASM+R1881	10.7	1.4	<0.0001
Lipofectin control+RT	3.9	0.8	0.219
AS+RT‡§	32.7	1.4	<0.0001
ASM+RT	9.6	1.6	<0.0001
AD+RT‡	7.2	1.0	1.000
AD+AS+RT§	40.4	2.5	<0.0001
AD+ASM+RT	16.2	3.0	<0.0001
AD+R1881+RT	5.3	1.1	0.0001
AD+R1881+AS+RT§	34.3	1.8	<0.0001
AD+R1881+ASM+RT	12.2	2.1	<0.0001

Abbreviations: AS = antisense MDM2; ASM = antisense mismatch; AD = androgen deprivation; RT = radiation therapy; SEM = standard error of the mean.

* Compared to group above, one-way ANOVA, Bonferroni test. The average of 4 experiments is shown.

† AD+AS vs. AS (p < 0.0001). AD+AS vs. AD+AS+R1881 (p = 0.018).

‡ AD+AS vs. AS+RT (p = 1.000); AD+AS vs. AD+RT (p < 0.0001).

§ AD+AS vs. AD+AS+RT (p = 0.106); AS+RT vs. AD+AS+RT (p < 0.0001); AD+AS+RT vs. AD+R1881+AS+RT (p = 0.964).

Overall cell death by clonogenic cell survival assay

Clonogenic cell survival experiments were performed to determine whether the added, but not significant, early cell killing from apoptosis due to AS+AD+RT translates into a significant increase in overall cell killing, i.e., the cell killing manifested over time. The early cell death measurements by trypan blue and the apoptosis markers may not be representative of all cell death occurring over time. Figure 2 shows the clonogenic assay results for LNCaP cells grown for 2–3 days in CM and then treated with lipofectin alone, AS, or ASM for 24 h before RT. The cells were then replated immediately after RT at 2, 4, or 6 Gy. The results show LNCaP radiosensitization by AS at all RT dose levels, over the CM and ASM controls. Figure 3 reveals that radiosensitization was further enhanced when AD was added to AS and that this effect was reduced by R1881 supplementation. The radiosensitizing action of AS+AD was much greater than the minor effect observed from ASM+AD.

DISCUSSION

Androgen deprivation and RT are central to the treatment of prostate cancer patients with high-risk prostate cancer. Even with the gains seen from this combination over single-modality therapy, the outcome of such high-risk patients remains rather poor. An understanding of the mechanisms of the interaction between AD and RT could lead to novel therapies that dramatically alter the failure profile.

Prior studies have indicated that p53 may have a role in the apoptotic response of prostate epithelial cells to AD (20). The results, however, have not been conclusive (21, 22). Little is known about why most prostate cancers respond to AD preferentially with a shift from cell proliferation to quiescence in the setting of minimal increases in apoptosis (23–28). There must be a key regulatory defect in the apoptotic pathway that preferentially shunts cells into quiescence instead of apoptosis. The data presented here point to MDM2. Of all of the proteins in the apoptotic pathway examined, MDM2 expression levels fluctuated in tandem with previously defined changes in apoptosis in response to AD+RT. We recently reported that in LNCaP cells grown *in vitro* (18) and in R3327-G Dunning rat prostate tumors grown *in vivo* (19), when AD precedes RT by 3 days, a supra-additive apoptotic response, over AD or RT given individually, is evidenced. Although supra-additive apoptosis was observed, the extent of the supra-additive response was rather minimal. The general lack of apoptosis seen in the response of prostate cancer cells to AD or RT alone, and

Table 4. Caspase 3+7 quantification of early apoptosis

Treatment	Mean	SEM	p*
Lipofectin control	114	16	–
AS†	335	19	<0.0001
ASM	199	25	0.169
AD	73	20	0.333
AD+AS†‡	504	7	<0.0001
AD+ASM	215	13	<0.0001
AD+R1881	109	29	1.000
AD+AS+R1881†	349	20	<0.0001
AD+ASM+R1881	170	33	0.006
Lipofectin control+RT	89	10	1.000
AS+RT‡§	547	46	<0.0001
ASM+RT	259	27	<0.0001
AD+RT‡	112	15	0.071
AD+AS+RT§	610	35	<0.0001
AD+ASM+RT	302	15	<0.0001
AD+R1881+RT	90	21	<0.0001
AD+R1881+AS+RT§	491	50	<0.0001
AD+R1881+ASM+RT	218	38	<0.0001

Abbreviations: AS = antisense MDM2; ASM = antisense mismatch; AD = androgen deprivation; RT = radiation therapy; SEM = standard error of the mean.

* Compared to group above, one-way ANOVA, Bonferroni test. The average of 3 experiments is shown.

† AD+AS vs. AS (p = 0.014); AD+AS vs. AD+AS+R1881 (p = 0.039).

‡ AD+AS vs. AS+RT (p = 1.000); AD+AS vs. AD+RT (p < 0.0001).

§ AD+AS vs. AD+AS+RT (p = 1.000); AS+RT vs. AD+AS+RT (p = 1.000); AD+AS+RT vs. AD+R1881+AS+RT (p = 0.571).

the modest short-lived increase in apoptosis from the combination, suggest that apoptosis is being suppressed.

Under the conditions of AD+RT, the relative levels of MDM2 increase, as compared to AD alone or AD+RT+R1881. In light of the increase in apoptosis levels observed herein when MDM2 expression is suppressed, it seems that the increase in MDM2 in response to AD+RT is due to feedback regulation, such that MDM2 dampens what would otherwise be a very pronounced apoptotic response in normal prostate epithelial cells. Because overexpression of MDM2 is seen in 30%–40% of prostate cancers, the action of MDM2 on response to AD and/or RT has significant clinical implications.

Previously we found that the suppression of MDM2, through the use of antisense MDM2 oligonucleotides (AS), not only induces significant levels of apoptosis in LNCaP cells by itself, but also results in a pronounced enhancement in apoptosis when combined with AD (17). Those findings have been substantiated and extended in this communication. The main question posed here was whether AS sensitizes cells to the combination of AD+RT when all of the other possible treatments are considered. Antisense MDM2 has been shown to sensitize tumor cells to radiation (13). Radiosensitization in terms of the apoptotic response by AS was confirmed in LNCaP cells. Both AD+AS and RT+AS displayed greater levels of apoptosis than the sum of the individual treatments. When all three treatments were combined, there was a consistent, albeit insignificant, increase in apoptosis seen over AD+AS or RT+AS. Because apoptosis was measured at a single point in time and may not be reflective of overall cell killing, clonogenic cell survival assays were performed.

By clonogenic assay, AS has been shown previously to

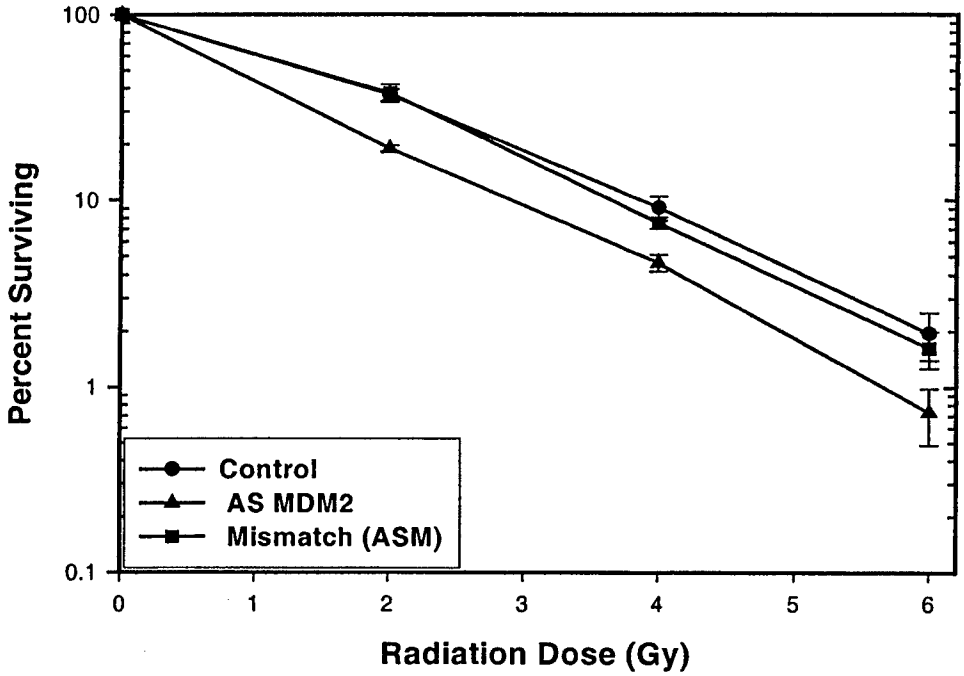

Fig. 2. Clonogenic assays of LNCaP cells cultured in CM alone or with AS or ASM (200 nM) added for 24 h before RT at 2, 4, or 6 Gy.

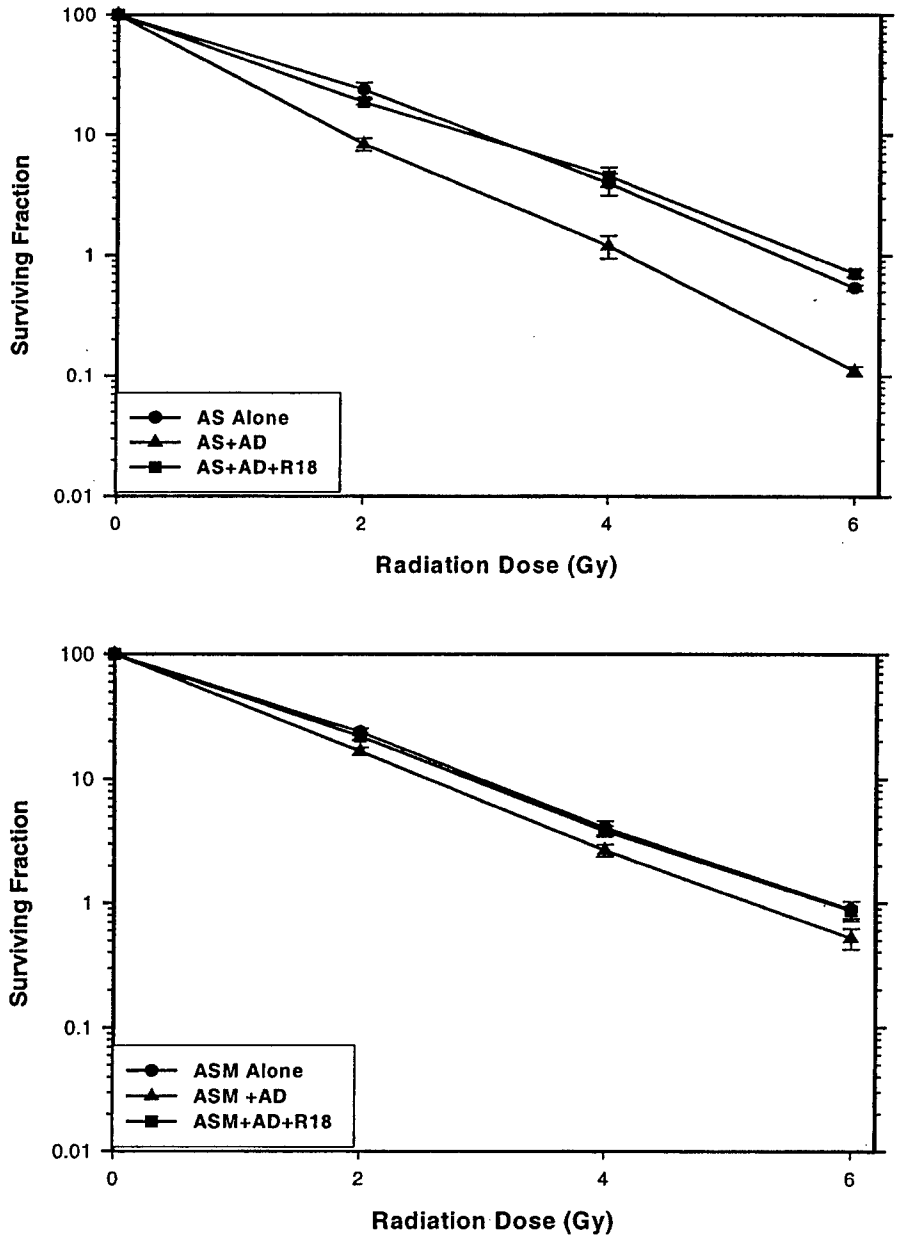

Fig. 3. Clonogenic assays of LNCaP cells cultured in CM or AD medium for 48–72 h and exposed to AS or ASM (200 nM) for 24 h before RT at 2, 4, or 6 Gy. AS = antisense MDM2; AD = androgen deprivation; R18 = synthetic androgen R1881; ASM = antisense mismatch.

significantly reduce clonogen survival when added to AD, as compared to each treatment applied individually. We show here that AS is also a potent radiosensitizer. Moreover, a further reduction of clonogen survival was evidenced when AS+AD+RT were given together, as compared to the controls (Fig. 3). The reduction in clonogenic cell survival was significant, and seemed to be greater than that observed by apoptosis alone. This could be related to the technical difficulty in summing apoptosis over time, which we did not attempt to do, or to other effects on cell survival, such as mitotic cell death. In either case, the data substantiate the critical role of MDM2 in the response of prostate cancer cells to AD and RT.

CONCLUSION

In summary, MDM2 is emerging as a central regulatory component in the cell death response of prostate cancer cells to AD and RT and has the potential to be manipulated therapeutically with AS. The hypothesized

mechanism for AS action is alteration of p53 expression via effects on MDM2 (the LNCaP cell line used is wild type for p53), although p53-independent effects may also contribute (7, 29). By enhancing the cell death response to AD, AS should improve cure rates by promoting cell death in micrometastatic deposits, as well as reduce the number of clonogens at the primary site. The reduction in clonogens from AD+AS, when combined with the radiosensitizing effects of AS, makes this strategy ideal for the man with high-risk prostate cancer.

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Antisense MDM2 Oligonucleotides Restore the Apoptotic Response of Prostate Cancer Cells to Androgen Deprivation

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BACKGROUND. Early in the malignant transformation of prostate epithelial cells, the apoptotic response to androgen deprivation (AD) is lost and the principle response is a slowing of cell growth. In this study, we tested whether interruption of MDM2 function using antisense MDM2 oligonucleotide (AS) affects the apoptotic response of prostate cancer cells to AD.
METHODS. Wild type LNCaP cells and MDM2-overexpressing (LNCaP-MST) cells were treated with AS alone or in combination with AD. Protein levels of MDM2, p53, and p21 were determined by Western blotting. Cell viability was measure by trypan blue staining. Apoptotic cell death was confirmed by cell morphological changes, annexin V/propidium iodide staining and caspase-3 + 7 activity. Overall cell survival was quantified by clonogenic assay.
RESULTS. AS inhibited MDM2 expression to a greater extent in LNCaP cells, as compared to LNCaP-MST cells. AS enhanced the expression of p53 and p21 in both cell lines. The growth inhibitory and cell death effects of AS + AD were generally greater than AS alone in LNCaP cells. Treatment of LNCaP cells with AS + AD for 72 hr caused a significant increase in cell death (66%) over AD alone (13%), AS alone (33%), or AD + AS + R1881 (34% with synthetic androgen replacement) that was attributable mainly to apoptosis. Clonogenic survival reflected the same pattern.
CONCLUSIONS. Our results suggest that the apoptotic response of prostate cancer to AD is strongly influenced by MDM2 expression. Antisense MDM2 has broad potential as a therapeutic agent to sensitize prostate cancer cells to AD therapy by enhancing apoptotic cell death. *Prostate 60: 187–196, 2004.* © 2004 Wiley-Liss, Inc.

KEY WORDS: **apoptosis; annexin V; caspase; clonogenic survival**

INTRODUCTION

Androgen deprivation (AD) is the most common treatment for advanced prostate cancer. Although this therapy successfully results in dramatic regression of prostate tumors, the responses are generally temporary, and eventually progression to androgen independence occurs in the vast majority of cases [1]. AD is effective at causing tumor shrinkage by promoting apoptosis and a shift to quiescence [2,3]. Although

Abbreviations: AD, androgen deprivation; AS, anti-MDM2 antisense; RT, radiation therapy; CM, completed medium; AD medium, charcoal stripped serum containing medium; R1881, synthetic androgen; LNCaP, human prostate cancer cell line that has wild-type expression of MDM2; LNCaP-MST, stably-transfected LNCaP cells which overexpress MDM2.

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normal prostate epithelial cells exhibit an extensive apoptotic response to AD [4], it appears that this response is appreciably reduced or lost early in malignant transformation [5–9]. The main effect of AD in these cases is a reduction in cell proliferation. If the apoptotic response of prostate cancer to AD could be restored through manipulation of the intracellular molecular milieu, it may be possible to enhance and prolong clinical responses, and perhaps ultimately patient survival.

MDM2 is an oncogene and the gene product is a key protein in the apoptotic pathway which binds to p53, E2F-1, and pRb, as well as other proteins [10]. It is induced by p53, binds to p53 with high affinity, and is a pivotal negative regulator of p53 action. The *MDM2* gene is amplified in a variety of human tumors [11], including prostate cancer [12–15]. The expression of MDM2 is increased in prostate cancer patients with high risk local–regional disease [16] and with the development of hormone refractory disease [9]. MDM2 is also a target for cancer therapy. Suppression of MDM2 using anti-MDM2 antisense (AS) sensitizes tumor cells to radiation [17] and chemotherapy [18–21].

Little is known about the molecular mechanisms that govern prostate cancer response to AD. Although functional p53 protein is not essential for the apoptotic response of normal prostate epithelial cells to castration [22,23], abnormal or suppressed p53 expression has been associated with resistance to AD [24–26]. Since MDM2 ablates p53 function, we hypothesized that MDM2 may contribute to the modulation of the apoptotic response to AD. In this study, the role of MDM2 was examined by suppressing MDM2 expression with a second generation antisense oligonucleotide (AS). The effects of AS were tested on wild type LNCaP and MDM2-overexpressing (LNCaP-MST) cells.

MATERIALS AND METHODS

Antisense Oligonucleotides

The antisense MDM2 20-mer mixed-backbone oligonucleotide (AS; 5′-UGACACCTGTTCTCACUCAC-3′) and its mismatch control oligonucleotide (ASM; 5′-UGTCACCCTTTTTCATUCAC-3′) were obtained from Hybridon, Inc. (Cambridge, MA). They were stored as frozen aliquots at −20°C.

Cell Culture and Transfection

LNCaP cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 medium, containing 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin–streptomycin (complete medium, CM), as described previously [27]. Cells were typically cultured for 24 hr in CM before the culture conditions were altered. AD was achieved by culture in 10% charcoal-stripped serum containing medium (AD medium). Replacement of androgen was done by adding the synthetic androgen R1881 (NEN Life Science Products, Boston, MA) at 1×10^{-10} M to AD medium [27].

To establish stable transfectants of MDM2 in LNCaP cells, cells were seeded at 1×10^6 cells per 10 cm dish for 24 hr and transfected with the pCMV-mdm2 expression plasmid by lipofectamine according to the manufacturer's procedure (Invitrogen, Carlsbad, CA). Neomycin-resistant cells were selected in the presence of 800 μg/ml Geneticin (G418, Life Technologies, Gaithersburg, MD) 48 hr after transfection. Expression of MDM2 was tested by Western blot analysis of cells obtained from a single colony.

Western Blot Analysis

Cells (1×10^6/dish) were incubated with 200 nM of AS or ASM in 4 ml culture medium for 24 hr in the presence of 7 μg/ml lipofectin (Invitrogen). Cells were lysed in a lysis buffer (50 mM Tris-Hcl, pH 6.8, 2% sodium dodecyl sulfate (SDS) with protease inhibitor cocktail set I (Calbiochem, San Diego, CA) and were sonicated. Protein concentration was determined using the BCA protein assay reagent kit (Pierce, Rockford, IL). Equal amounts of protein (50 μg) were separated by SDS–PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were incubated with blocking buffer (PBS containing 0.1% Tween-20 and 5% non-fat milk) for 1 hr at room temperature and were washed twice in washing buffer (PBS containing 0.1% Tween-20) for 5 min. Membranes were immunoprobed with either anti-MDM2 monoclonal antibody (mAb) at 1:1,000, anti-p53 mAb at 1:1,000, anti-p21 mAb at 1:1,000, or anti-β actin at 1:5,000 dilution (Calbiochem), incubating overnight at 4°C. Membranes were then washed and incubated with 1:2,000 diluted sheep antimouse IgG horseradish peroxidase conjugated secondary antibody (Amersham Life Science) for 1 hr at room temperature. After washing, the proteins were detected by the enhanced chemiluminescence reagents according the manufacturer's direction (Amersham, Aylesbury, UK).

Cell Growth and Cell Viability

For the cell growth experiments, cells were plated in 24-well plates at 2.5×10^4 cells/well and cultured in CM for 24 hr. The medium was then changed and replaced with either CM, AD medium, or AD + R1881 medium. Simultaneous with the medium change, 20 nM of AS or ASM was added into medium in the

presence of lipofectin (7 μg/ml). The number of viable cells was counted at 24 hr intervals.

For the cell viability assay, 5×10^4 cells/well were seeded in 24-well plates. Cells were treated with 200 nM of AS or ASM and the percentage of dead cells measured by trypan blue dye exclusion at various times.

Confirmation of Apoptosis

Two methods were used to confirm apoptotic cell death. LNCaP cells were cultured in either CM, AD medium, or AD + R1881 medium for 48 hr. Cells were then incubated with 200 nM AS or ASM in 4 ml of medium for 24–48 hr in the presence of lipofectin (7 μg/ml). Cell morphological changes were examined by light microscopy.

Annexin V staining was used to determine the proportion of apoptotic cells. After incubation with 200 nM AS or ASM, all cells (floating and attached) were harvested by trypsinization, labeled with annexin V-PE and 7-amino-actinomycin D (7-AAD) (Guava Technologies, Inc., Burlingame, CA) according to the manufacturer's instructions and analyzed by flow cytometry on a GuavaPC personal flow cytometer.

Caspase-3 + 7 activity was measured using a fluorometric substrate, Z-DEVD-Rhodamine (The Apo-ONE™ Homogeneous Caspase-3/7 Assay kit; Promega, Madison, WI). Cells were cultured in CM, AD, or AD + R1881 medium for 2 days and then incubated with AS or ASM (200 nM) for 30 hr. A total of 5×10^4 cells in 100 μl culture medium were mixed with 100 μl of Homogeneous Caspase-3/7 reagent in 96-well plates and incubated at room temperature for 18 hr. Substrate cleavage was quantified fluorometrically at 485 excitation and 538 nm emission. Fluorescence was measured on a fluorescent plate reader (LabSystems, Inc., Franklin, MA). As a control, caspase-3 + 7 activity was inhibited in some cells by adding Ac-DEVD-CHO (Promega) to the culture before the assay.

Clonogenic Assay

Clonogenic assays were performed as described previously [28]. In brief, cells were cultured in CM, AD medium, or AD + R1881 medium for 48 hr and then incubated with AS or ASM at various concentrations for 24 hr in presence of lipofectin (7 μg/ml). The cells were trypsinized, serially diluted, and known numbers of cells replated into 100 mm dishes. The plates were incubated for 12–14 days and stained with 0.25% methylene blue. The number of colonies was determined using an automated counter (Imaging products International, Inc., Chantilly, VA). The clonogenic survival results were corrected for differences in plating efficiency from the various culture conditions. The dilutions for clonogenic assay were done in triplicate and the results were averaged together (intra-experiment averages). The data shown in the clonogenic survival table represent an average from multiple experiments (inter-experiment averages).

RESULTS

Effect of AS on MDM2, p53, and p21 Protein Levels

To evaluate the effects of MDM2 suppression and overexpression on AD, stable transfectants of LNCaP cells that overexpressed human MDM2 were established. As displayed in Figure 1, expression of MDM2 was increased substantially in LNCaP-MST cells, compared to parental LNCaP cells.

The effects of AS on MDM2, p53, and p21 expression in both LNCaP and LNCaP-MST cells were measured. Figure 1 shows that AS decreased the expression of MDM2 and increased the expression of both p53 and p21 in a dose-dependent manner in LNCaP and LNCaP-MST cells. There appears to be less of a reduction of MDM2 expression with high concentrations of AS (500 nM) in LNCaP-MST cells; although, this could be related to preferential cell death and loss of the subpopulation of cells with low MDM2 levels. Treatment with ASM appeared to have a slight stimulatory effect on MDM2 expression and little effect on p53 and p21 protein levels, even at 500 nM.

Studies were then performed to determine the action of AS on LNCaP and LNCaP-MST cells grown in vitro in androgen deprived (AD) medium. After 2 days of culture in either CM, AD medium, or AD + R1881 medium, cells were incubated with 200 nM AS or ASM in the presence of lipofectin for 24 hr. As illustrated in Figure 1C, AD alone caused a reduction in MDM2, p53, and p21 protein levels in LNCaP cells. When AS was combined with AD, MDM2 expression in LNCaP cells was further reduced and the expression of p53 and p21 remained high. When R1881 was added to LNCaP cells treated with AD, there was little alteration in MDM2, p53, and p21 levels from the addition of AS or ASM, over that seen with AD alone. Figure 1D shows that AD caused slight reductions in MDM2 and p53 levels, and a larger reduction in p21 expression in LNCaP-MST cells. Treatment of LNCaP-MST cells with AS + AD further reduced MDM2 levels, while the expression of p53 and p21 remained high. When R1881 was added to LNCaP-MST cells treated with AD, there was little alteration in MDM2, p53 and p21 levels from the addition of AS or ASM, over that seen with AD alone.

LNCaP and LNCaP-MST Cell Growth and Viability After AS ± AD Treatment

Previous studies have shown that the growth of LNCaP cells is inhibited considerably when cultured in

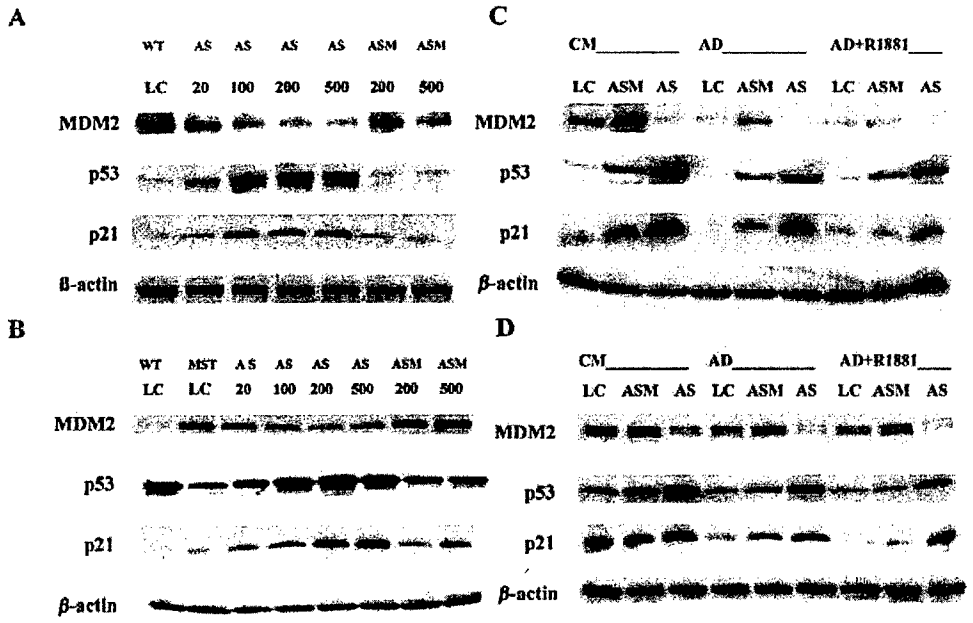

Fig. 1. Western blot analysis of MDM2, p53, and p21 levels in LNCaP (WT, **panel A**) and LNCaP-MST (MST, **panel B**) cells exposed to different concentrations of AS (in nM) for 24 hr. Western blot analysis of MDM2, p53, and p21 levels in cells grown in complete, AD, and AD + R1881 medium is shown in **panel C** for LNCaP and **panel D** for LNCaP-MST cells. Total protein was 30 μg per lane. LC, lipofectin control; AD, androgen deprivation; AS, antisense MDM2.

AD medium and is partially reversed by adding the synthetic androgen R1881 back to AD medium [27]. To evaluate whether AS combined with AD resulted in enhanced LNCaP cell growth inhibition over AS or AD given individually, different doses of AS (10–200 nM) were first tested. Cell growth inhibition was dose-dependent (data not shown). LNCaP cells were then treated with 20 nM AS or ASM with or without AD for 4 days. The findings depicted in Figure 2A illustrate that the replication of LNCaP cells was completely suppressed after exposure to AS + AD; the combination resulted in more growth inhibition than the individual treatments with AS or AD ($P < 0.001$, one way analysis of variance, Bonferroni test at day 3 of culture). The synthetic androgen R1881 partially restored the growth of LNCaP cells treated with AS + AD such that cell number at day 3 was higher with R1881 added, as compared to AS + AD alone ($P = 0.001$, Bonferroni test). The growth inhibitory effects of AS, AD, and AS + AD were less overall in

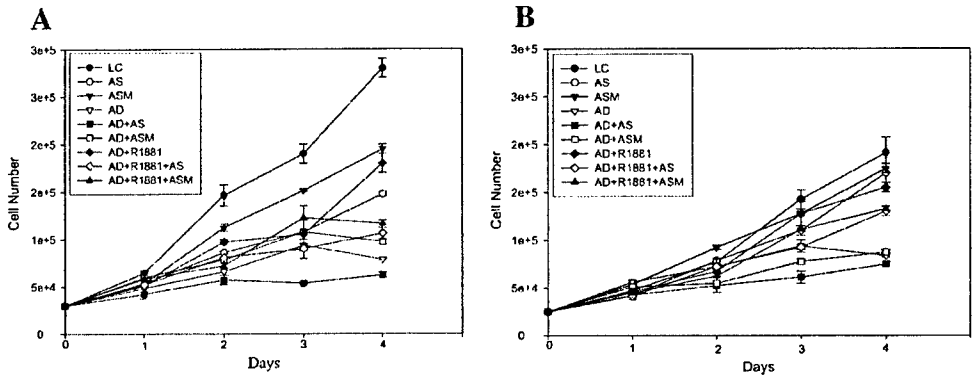

Fig. 2. LNCaP (**A**) and LNCaP-MST (**B**) cell growth inhibition in vitro by AS or ASM in combination with AD or AD + R1881. Cells were cultured in complete medium (CM) for 24 hr, then the medium was changed and the cells cultured further in CM, AD, or AD + R1881 medium. Simultaneously 20 nM AS or ASM was added to medium in presence of lipofectin. The number of viable cells were counted at the indicated times. LC, lipofectin control.

LNCaP-MST cells; although the general pattern for the individual and combined treatments was similar (Fig. 2B).

Cell Death From AS ± AD

The early overall cell death response of LNCaP cells to AS, AD and the combination was first examined using trypan blue dye uptake. The cells were exposed to 200 nM AS with or without AD for different periods of time. A summary of three experiments measuring LNCaP cell death after 72 hr is shown in Table I. Cell death from AS + AD was significantly enhanced (66%) over that of ASM + AD (22%), AS alone (33%), or AD alone (13%). The cell killing effect of AS + AD was significantly reversed to 34% when the synthetic androgen R1881 was added. Table II displays the cell death effects of AS, AD and the combination on LNCAP-MST cells. While there was a significant increase in cell death from AS + AD over AS or AD alone, and this effect was reversed by R1881, the extent of these differences was less than was seen in the LNCaP line. The proportions of cells dying after incubation of LNCaP and LMCaP-MST cells in lipofectin only or AD alone were comparable.

Figure 3 shows the early cell death response of LNCaP cells to AD alone, ASM + AD, and AS + AD in terms of the morphological effects, which are suggestive of apoptosis. These effects included decreased cell density, elongation and shrinkage of cells by 24 hr, rounding of some cells, and detachment from the culture surface by 48 hr. These morphological changes were more pronounced from AS + AD than from AD alone or ASM + AD.

TABLE I. Effect of Antisense MDM2 on LNCaP Cell Death Measured by Trypan Blue Dye Uptake

Treatment	Mean	SEM	P*
Lipofection control	5.8	0.7	—
AS (Antisense MDM2)	32.8	1.5	<0.0001
ASM (mismatch control)	15.7	0.2	<0.0001
AD (androgen deprivation)	13.5	0.3	1.000
AD + AS**	66.0	1.5	<0.0001
AD + ASM	21.8	2.7	<0.0001
AD + R1881	7.5	1.3	<0.0001
AD + AS + R1881	34.0	0.8	<0.0001
AD + ASM + R1881	16.5	0.8	<0.0001

Percentage of cell death measured by trypan blue dye uptake after 3 days of culture. The data shown represent the average values (±SEM) from three independent experiments.
*Compared to group above, one way Anova, Bonferroni test.
**Other comparisons: AD + AS versus AD + AS + R1881 ($P < 0.0001$); AD + AS versus AS ($P < 0.0001$).

TABLE II. Effect of Antisense MDM2 on LNCaP-MST Cell Death Measured by Trypan Blue Dye Uptake

Treatment	Mean	SEM	P*
Lipofection control	6.3	0.9	—
AS (antisense MDM2)	19.0	4.6	0.003
ASM (mismatch control)	12.3	0.9	0.604
AD (androgen deprivation)	12.3	1.5	1.000
AD + AS**	32.0	1.2	<0.0001
AD + ASM	17.0	0.6	<0.0001
AD + R1881	10.0	1.2	0.456
AD + AS + R1881	19.0	0.6	0.081
AD + ASM + R1881	13.7	0.9	1.000

Percentage of cell death measured by trypan blue dye uptake after 3 days of culture. The data shown represent the average values (±SEM) from three independent experiments.
*Compared to group above, one way Anova, Bonferroni test.
**Other comparisons: AD + AS versus AD + AS + R1881 ($P = 0.002$); AD + AS versus AS ($P = 0.002$).

Direct measurements of apoptosis were performed to determine the contribution of apoptosis to early overall cell death from AS ± AD. Apoptosis was measured directly by annexin V binding. The cells were cultured in either complete, AD, or AD + R1881 medium for 48 hr and then incubated with AS or ASM for 48 hr. Table III displays the results of annexin V staining in LNCaP cells, revealing that the cells undergoing early apoptosis (annexin-V-PE-positive and 7-AAD-negative) increased to 26.5% after AS + AD AD treatment, relative to 17.5 and 8.5% for AS and AD given individually. The difference was significant for AS + AD versus AD alone, but not for AS + AD versus AS alone (Table III). Table IV shows that a similar trend was observed for the effect of AS on the apoptosis of LNCaP-MST cells; however, the addition of AD to AS did not result in an enhanced response over AS alone. The lack of sensitization of LNCaP-MST cells to AS + AD could be related to the greater amount of apoptosis seen with AS alone (Table IV), as compared to LNCaP cells (Table III). A similar pattern was apparent in the caspase-3 + 7 experiments (Tables V and VI).

Caspase-3 + 7 activity is another marker of apoptosis that was used to corroborate the annexin V findings. The caspase data reflected that obtained with annexin V staining. After AS exposure, caspase-3 + 7 activity was higher in LNCaP-MST cells (Table VI) than in LNCaP cells Table V). In LNCaP cells, AS + AD resulted in significantly elevated caspase-3 + 7 levels, compared to AS and AD applied individually (Table V). Moreover, caspase-3 + 7 activity was inhibited by the addition of R1881 to AS + AD to approximatley the levels of AS alone. The addition of specific caspase inhibitor Ac-DEVD-CHO (data not shown) reduced caspase-3 + 7

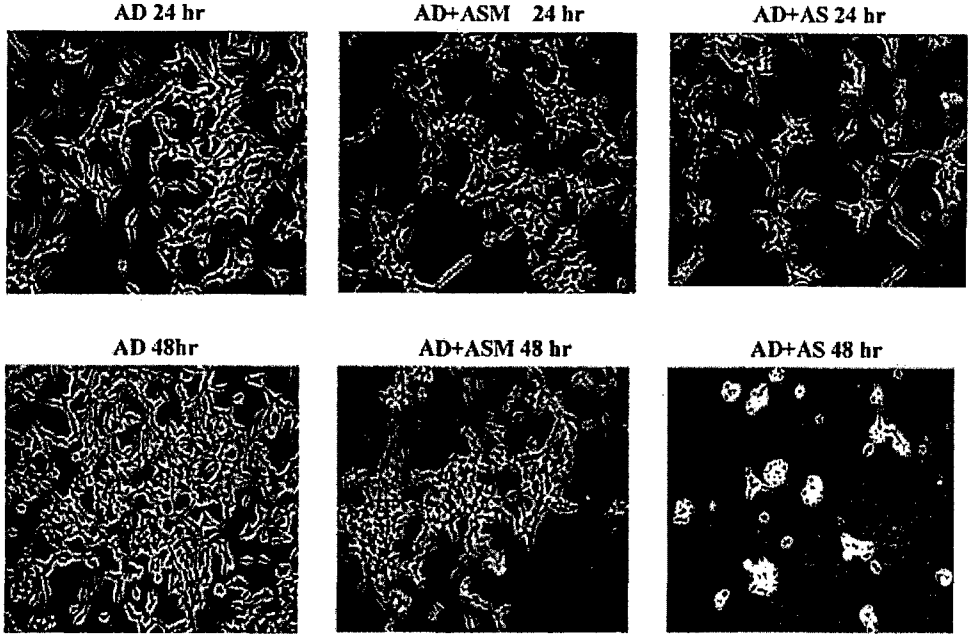

Fig. 3. Morphology of LNCaP cells after treatment for 24 and 48 hr with AS or ASM (200 nM), and AD or AD + R1881. The cells were examined under light microscopy magnification of 200×.

activity. In LNCaP-MST cells, AS caused a significant increase in apoptosis over ASM and the lipofectin control, but the addition of AD to AS did not result in a further increase in apoptosis (Table VI). These results suggest that AS restores LNCaP tumor cell apoptosis to AD through p53 by activating caspase-3 + 7, and that the overexpression of MDM2 inhibits or delays this action. The data also indicate that elevated MDM2 levels, as seen in LNCaP-MST cells, result in a more pronounced apoptotic response to AS, and that under such conditions the addition of AD does not result in more apoptosis.

Overall Cell Death by Clonogenic Assay

Clonogenic survival experiments were performed to determine whether the added early cell killing from

TABLE III. Effect of Antisense MDM2 on LNCaP Cell Apoptosis Measured by the Annexin V-Assay

Treatment	Mean	SEM	P*
Lipofectin control	4.9	1.3	—
AS (antisense MDM2)	17.5	1.2	0.02
ASM (mismatch control)	8.3	0.9	0.264
AD (androgen deprivation)	8.5	1.6	1.000
AD + AS**	26.5	2.7	<0.0001
AD + ASM	13.0	2.2	0.012
AD + R1881	5.4	1.0	0.826
AD + AS + R1881	15.2	4.2	0.174
AD + ASM + R1881	8.5	2.1	1.000

LNCaP cells were treated for 48 hr with AS-MDM2 (200 nM) alone or in combination with AD ± R1881 and the percentage of apoptotic cells measured by flow cytometric analysis of annexin V-PE and 7-AAD staining. The data shown represent the average values (±SEM) from three independent experiments.
*Compared to group above, one way Anova, Bonferroni test.
**Other comparisons: AD + AS versus AD + AS + R1881 ($P = 0.061$); AD + AS versus AS alone ($P = 0.32$).

TABLE IV. Effect of Antisense MDM2 on LNCaP-MST Cell Apoptosis Measured by the Annexin V-Assay

Treatment	Mean	SEM	P*
Lipofectin control	3.8	0.7	—
AS (antisense MDM2)	35.0	1.6	<0.0001
ASM (mismatch control)	16.8	1.7	0.002
AD (androgen deprivation)	10.3	1.8	1.000
AD + AS**	35.3	3.6	<0.0001
AD + ASM	21.8	3.6	0.035
AD + R1881	7.3	1.3	0.018
AD + AS + R1881	32.4	3.4	<0.0001
AD + ASM + R1881	20.0	2.0	0.073

LNCaP–MST cells were treated for 48 hr with AS-MDM2 (200 nM) alone or in combination with AD ± R1881 and the percentage of apoptotic cells measured by flow cytometric analysis of annexin V-PE and 7-AAD staining. The data shown represent the average values (±SEM) from three independent experiments.
*Compared to group above, one way Anova, Bonferroni test.
**Other comparisons: AD + AS versus AD + AS + R1881 ($P = 1.000$); AD + AS versus AS alone ($P = 1.000$).

TABLE V. Effect of Antisense MDM2 on LNCaP Cell Caspase-3 + 7 Activity

Treatment	Mean	SEM	P*
Lipofectin control	85	9	—
AS (antisense MDM2)	357	29	<0.0001
ASM (mismatch control)	181	29	0.020
AD (androgen deprivation)	48	7	0.186
AD + AS**	635	64	<0.0001
AD + ASM	121	26	<0.0001
AD + R1881	115	13	1.000
AD + AS + R1881	430	36	<0.0001
AD + ASM + R1881	235	6	0.007

Caspase-3 + 7 activity was measured by fluorometric assay. LNCaP cells were cultured for 2 days with or without AD (±R1881) and then for 30 hr with 200 nM AS or ASM. The data shown represent the average values (±SEM) from three independent experiments.
*Compared to group above, one way Anova, Bonferroni test.
**Other comparisons: AD + AS versus AD + AS + R1881 ($P = 0.004$); AD + AS versus AS alone ($P < 0.0001$).

apoptosis due to AS + AD translates into a significant increase in overall cell killing. LNCaP and LNCaP-MST cells were cultured in CM, AD, or AD + R1881 medium for 2 days prior to exposure to AS for 24 hr before plating for clonogenic survival. As shown in Table VII, the percentages of LNCaP cells surviving were 59.7% for AS alone, 27.2% for AS + AD, and 54.7% for AS + AD + R1881. The difference between AS and AS + AD was significant. The percentage of LNCaP cells surviving after AS + AD + R1881 was reduced to

TABLE VI. Effect of Antisense MDM2 on LNCaP-MST Cell Caspase-3 + 7 Activity

Treatment	Mean	SEM	P*
Lipofectin control	211	12	—
AS (antisense MDM2)	966	19	<0.0001
ASM (mismatch control)	617	24	0.001
AD (androgen deprivation)	231	22	<0.0001
AD + AS**	879	55	<0.0001
AD + ASM	357	55	<0.0001
AD + R1881	234	4.9	1.000
AD + AS + R1881	697	82	<0.0001
AD + ASM + R1881	345	41	<0.0001

Caspase-3 + 7 activity was measured by fluorometric assay. LNCaP-MST cells were cultured for 2 days with or without AD (±R1881) and then for 30 hr with 200 nM AS or ASM. The data shown represent the average values (±SEM) from three independent experiments.
*Compared to group above, one way Anova, Bonferroni test.
**Other comparisons: AD + AS versus AD + AS + R1881 ($P = 0.251$); AD + AS versus AS alone ($P = 1.000$).

that seen with AS alone. For the LNCaP-MST cell line, no significant difference was observed between AS and AS + AD treatment.

DISCUSSION

Although AD is quite effective at reducing prostate cancer tumor burden and causing dormancy, an improvement in patient survival from AD treatment as a single agent has not been conclusively established. Early in the malignant transformation of prostate epithelial cells, the apoptotic response to AD is dramatically reduced or lost, and the principal response is a slowing of cell growth and reduction in cell proliferation [5–9]. Previously we found that R3327-G rat prostate tumors grown in vivo have a pronounced shift to quiescence in response to AD, with a minimal increase in apoptosis [3]. The human tumor LNCaP line responds similarly [27]. We hypothesized that if the apoptotic response of prostate cancer to AD could be restored through alterations in the molecular milieu, it may be possible to enhance overall tumor cell death. Such a strategy could then prolong clinical responses and perhaps ultimately patient survival. The oncogene MDM2 was selected as a target because it contributes significantly to the control of p53 function and preliminary western blot studies (not shown) revealed that MDM2 expression was tied to the apoptotic response of LNCaP cells to the combination of AD + RT.

MDM2 is a key protein in the apoptotic pathway that functions by negatively regulating p53 transcription directly and indirectly through effects on nuclear transport and p53 degradation. As a result, MDM2 interferes with normal growth control by preventing apoptosis [29]. Previous studies have shown that the suppression of MDM2 expression by AS increases the apoptotic response of some tumor cells to radiation or chemotherapy [15,17–21].

In the results presented here, AS specifically inhibited MDM2 expression in both LNCaP and MDM2 overexpressing LNCaP-MST cells. In parallel with this inhibition, p53 and p21 expression were enhanced. These increases were expected since MDM2 is a feedback regulator of p53. The incubation of cells in AD medium resulted in a decrease in MDM2 expression (LNCaP > LNCaP-MST). When the cells were cultured in AD medium with AS, there was a further abrogation of MDM2 expression in both cell lines. The suppression of MDM2 under the conditions of AS + AD resulted in proportionally increased tumor cell growth inhibition in vitro and significantly enhanced cell death responses (LNCaP > LNCaP-MST cells).

The major relative differences in the LNCaP and LNCaP-MST cell death responses were seen when

TABLE VII. Effects of AS on LNCaP and LNCaP-MST Cell Clonogenic Survival When Cultured in Complete, Androgen Deprived (AD) or AD + R1881 Medium

	% Clonogenic survival			
	LNCaP		LNCaP-MST	
Treatment	M ± SEM	P*	M ± SEM	P*
ASM	85.4 ± 0.9	—	88.7 ± 2.4	—
AS	59.7 ± 7.5	0.015	67.6 ± 9.4	1.000
AD + ASM	64.2 ± 3.0	1.000	70.9 ± 7.0	1.000
AD + AS**	27.2 ± 5.7	0.001	55.0 ± 11.2	1.000
AD + ASM + R1881	77.8 ± 1.1	<0.0001	83.5 ± 3.1	0.518
AD + AS + R1881	54.7 ± 2.6	0.033	61.5 ± 12.4	1.000

*Compared to group above , one way Anova, Bonferroni test, done separately for the six LNCaP and six LNCaP-MST groups. The data shown represent the mean (M) values (±SEM) from three independent experiments.
**Other LNCaP comparisons (n = 6 groups): AD + AS versus AD + AS + R1881 ($P = 0.009$); AD + AS versus AS alone ($P < 0.002$). Other LNCaP-MST comparisons (n = 6 groups): AD + AS versus AD + AS + R1881 ($P = 1.000$); AD + AS versus AS alone ($P = 1.000$).

AS was combined with AD. There was little disparity after treatment with lipofectin or AD alone in trypan blue uptake or apoptosis between LNCaP and LNCaP-MST cells. However, the LNCaP and LNCaP-MST lines responded differently to AS, AS + AD, and AS + AD + R1881. Overall early cell death by trypan blue uptake in LNCaP-MST cells incubated with AS + AD was greater than AS alone (Table II), but was half that seen in LNCaP cells (Table I). In contrast, the apoptotic response of LNCaP-MST cells to AS alone was greater than that of LNCaP cells; yet, there was no additional cell death from the combination of AS + AD. Wild type LNCaP cells had less of an apoptotic response to AS alone and a supra-additive response to AS + AD (Tables III–VI).

The discrepancy in the features of the trypan blue and apoptotic responses of LNCaP-MST cells to AS and AS + AD may be related to the intrinsic differences in the assays. Trypan blue uptake was measured over 3 days and included death from necrosis and apoptosis. The apoptosis assays were run over a shorter period of time and represent more of a snapshot of this form of cell death. Since cell death is a dynamic process, wherein cell death and disintegration may not be constant over time, some variation between assays is expected. Nonetheless, the early high apoptotic response of LNCaP-MST cells to AS and the lack of any added effect from AD was notable; this pattern was confirmed in both the annexin V and caspase-3 + 7 assays. One explanation is that the condition of MDM2 overexpression, which negatively affected p53 expression, created an enhanced sensitivity to MDM2 suppression by AS, resulting in higher levels of apoptosis than that seen in wild type LNCaP cells.

When the synthetic androgen R1881 was added back to the AD medium and LNCaP cells exposed to AS (AS + AD + R1881), cell death was reduced to approximately the levels seen with AS alone. The supra-additive cell death from AS + AD was clearly related to an interaction that appeared to result in the restoration of the apoptotic response to AD. More importantly, the same supra-additive effect was observed by clonogenic survival assay.

Wild type LNCaP cells displayed a supra-additive reduction in clonogenic survival to AS + AD, when compared to AS or AD given individually. The effect of AS + AD was reversed by adding R1881. Such a supra-additive decrement in clonogenic survival was not seen with LNCaP-MST cells. Thus, the blunted response of LNCaP-MST cells to AS + AD over that of AS alone was observed in both apoptotic (Tables IV and VI) and clonogenic survival (Table VII) assays; although, a significant increase in cell death from AS + AD over AS alone was seen for trypan blue dye uptake (Table II). As mentioned above, the trypan blue uptake and apoptosis assays measure early cell death response. Clonogenic survival sums cell death effects over time and is the more important assay. Thus, MDM2 proved to be central to the response of both cell lines to AD. MDM2 suppression in the setting of wild type LNCaP cells appeared to restore the cell death response to AD that was lost during transformation. MDM2 overexpression in LNCaP-MST cells resulted in a significant reduction in the interaction of AS with AD, confirming that MDM2 is an important determinant of the cell death response of prostate cancer cells to AD.

The molecular mechanisms that govern the response of androgen sensitive prostate cancer cells to AD alone

are unclear. There is primarily indirect evidence that abnormal p53 expression may contribute to the development of androgen insensitivity; this evidence is derived mainly from analyses of tumor tissue from patients with androgen insensitive prostate cancer [24,25,30]. Burchardt et al. [26] have shown more directly that the suppression of p53 function, using antisense p53, resulted in LNCaP tumor cell growth in castrated nude mice that did not occur when p53 was expressed. In addition, there are data suggesting that p21 is an important determinant of prostate cancer androgen insensitivity [31–33]. Antisense MDM2 has a downstream effect on p21 through p53. Recent data also suggest that antisense MDM2 promotes apoptosis in vitro and tumor growth inhibition in vivo independent of p53 status [15,34,35]. Thus, MDM2 appears to have p53 dependent and independent pathways that promote tumor resistance to treatments such as radiation, chemotherapy, and as shown in this report, AD.

In conclusion, our data reveal for the first time that MDM2 expression has a key role in the early (apoptotic) and overall (clonogenic) cell death response of prostate cancer to AD. The supra-additive cell killing from AS + AD was reversed by androgen replacement, suggesting that AS restored the apoptotic response of LNCaP cells. The clinical implications are considerable. Antisense MDM2 should impact on the full spectrum of patients with prostate cancer. Patients with advanced metastatic disease would have more pronounced and probably lasting responses to AD. Intermediate and high risk prostate cancer patients with clinically localized (no overt evidence of metastasis) stand to benefit from more complete eradication of micrometastatic disease from the addition of AS to sensitize cells to AD and local disease from the addition of AS to sensitize cells to radiation. Patients with early disease treated with radiotherapy might gain from the use of AS to lower radiation doses needed for cure. The application of AS to the combination AD + RT is currently under investigation.

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Ki-67 Staining Index Predicts Distant Metastasis and Survival in Locally Advanced Prostate Cancer Treated With Radiotherapy: An Analysis of Patients in Radiation Therapy Oncology Group Protocol 86-10

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ABSTRACT

Purpose: **Proliferative activity defined by Ki-67 staining index (SI) has been correlated with progression and prognosis in a number of malignant tumors including prostate cancer. However, few studies have examined Ki-67 SI in pretreatment diagnostic material from patients treated with definitive radiotherapy. In a prior study, we found that a Ki-67 SI of >3.5% was associated with poorer patient outcome. The goals of this analysis were to validate the prognostic value of Ki-67 SI and this cut point.**

Experimental Design: **Of 456 assessable patients in Radiation Therapy Oncology Group Protocol 86-10, diagnostic material from 108 patients was available for Ki-67 analysis using MIB-1 antibody. Sixty patients were treated with external beam radiotherapy (EBRT) alone, and 48 patients were treated with short-term androgen deprivation + EBRT. Median follow-up was 9 years for those living. The relationship of Ki-67 with distant metastasis (DM), disease-specific survival (DSS), and overall survival (OS) was examined.**

Results: **The median Ki-67 SI was 7.1% (range, 0.2–45.5%). The 7.1% cut point was associated with DM and DSS; however, the 3.5% cut point was as strong a determinant and was the focus of this analysis. In Cox proportional hazards regression, Ki-67 SI was independently associated with DM and DSS. When the Ki-67 SI was ≤3.5% and >3.5%, the 5-year risk of DM was 13.5% and 50.8% ($P = 0.0005$), respectively, and the 5-year risk of DSS was 97.3% and 67.7% ($P = 0.0039$), respectively. No association of Ki-67 SI with OS was observed.**

Conclusions: **Higher Ki-67 SI was significantly associated with a greater risk of DM and DSS in locally advanced prostate cancer after definitive EBRT or AD + EBRT.**

INTRODUCTION

Quantification of the proportion of cells with nuclear Ki-67 antigen expression is a measure of growth fraction (1–4) and hence biological aggressiveness in malignancy. This immunohistochemically detected marker has shown potential as an independent correlate of outcome for prostate cancer patients treated with radical prostatectomy in the vast majority of reports (5–9). More recently, Ki-67 staining has shown promise for patients treated definitively with external beam radiotherapy (EBRT; Refs. 10–12). Using MIB-1 monoclonal antibody for the determination of Ki-67 activity in archival, formalin-fixed diagnostic material, Cowen *et al.* (12) found that when the proportion of tumor cells staining for Ki-67 [Ki-67 staining index (SI)] was >3.5%, biochemical and disease failure was significantly higher than when Ki-67 SI was ≤3.5%. Because a number of Ki-67 SI cut points have been described in different study populations, the applicability of one particular cut point and, consequently, the results of immunohistochemical staining of Ki-67 in clinical practice remain questionable.

In this report, the value of the 3.5% Ki-67 SI cut point was evaluated in a cohort of locally advanced patients treated in Radiation Therapy Oncology Group (RTOG) Protocol 86-10 (13–15). In this randomized trial of 456 assessable patients, half the patients received EBRT alone, and the other half received short-term neoadjuvant androgen deprivation (AD) + EBRT. The AD was started 2 months before and continued for the duration of EBRT. There were 108 cases available for the Ki-67 SI analyses described here.

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Table 1 Characteristics of patients by Ki-67 SI[a] at the 7.1% and 3.5% cut points

Characteristics	Ki-67 SI ≤ 7.1% (*N* = 55)	Ki-67 SI > 7.1% (*N* = 53)	*P*[b]	Ki-67 SI ≤ 3.5% (*N* = 37)	Ki-67 SI > 3.5% (*N* = 71)	*P*[b]
Age (yrs)						
<75	35 (64%)	44 (83%)	0.02	23 (62%)	56 (79%)	0.06
≥75	20 (36%)	9 (17%)		14 (38%)	15 (21%)	
Gleason score						
2–6	22 (41%)	5 (9%)	0.0002	17 (47%)	10 (14%)	0.0002
7–10	32 (59%)	48 (91%)		19 (53%)	61 (86%)	
Clinical category						
T_2	16 (30%)	15 (28%)	0.92	11 (30%)	20 (28%)	0.86
T_3	39 (70%)	38 (72%)		26 (70%)	51 (72%)	
Assigned treatment						
EBRT alone	34 (62%)	26 (49%)	0.18	18 (49%)	42 (59%)	0.29
EBRT + AD	21 (38%)	27 (51%)		19 (51%)	29 (41%)	

[a] SI, staining index; EBRT, external beam radiation therapy; AD, androgen deprivation.
[b] χ^2 statistics.

MATERIALS AND METHODS

Patient Characteristics. The details of RTOG Protocol 86-10 have been described previously (13). Of the 108 patients for whom pretreatment diagnostic material was available for Ki-67 SI analysis, 72 samples were from needle biopsies, and 36 samples were from transurethral resections. Confirmation of the pathological diagnosis and Gleason grading were done by the study pathologist (D. J. G.). A Gleason score of 7–10 was observed in 80 cases, and a Gleason score of <7 was observed in 27 cases (Gleason score was not available in 1 case). Clinical category T_2 was present in 31 patients, and T_3 was present in 77 patients. Radiotherapy alone (EBRT) was administered to 60 patients, and 48 cases received AD + EBRT (see Table 1).

Ki-67 Immunohistochemical Staining Procedure and Quantification. Paraffin-embedded archival sections (4 μm) were deparaffinized in xylene and then rehydrated in graded ethanol. Slides were subsequently heated in 10 mM citrate buffer (pH 6.0) for 50 min using a pressure cooker (BioCare Medical, Walnut Creek, CA). The slides were treated with 0.3% hydrogen peroxide for 5 min and then incubated with the monoclonal antibody MIB-1 (DAKO Corp., Carpinteria, CA) for 10 min. Secondary biotinylated antibody was then applied for 10 min, followed a 10-min incubation with streptavidin peroxidase (DAKO Corp.). After rinsing, slides were stained with diaminobenzidine chromogen solution (ResGen Invitrogen Corp., Carlsbad, CA) and counterstained with routine hematoxylin. Staining was accomplished in three batches, using a DAKO Autostainer (DAKO Corp.). Negative controls for the staining were biopsy slides stained with omission of the primary antibody. Positive controls were normal tonsil sections that had previously been studied by flow cytometry to determine the percentage of proliferating cells.

Nuclear staining of Ki-67 was considered positive. The Ki-67 SI was defined as the percentage of positive nuclei of a total of 2000 tumor cells counted using an eyepiece grid. The positive nuclei were counted by one investigator (R. L.) without prior knowledge of the patient prognosis-related information.

Definition of End Points. The end points investigated in the analyses were local failure, distant metastasis (DM), overall survival (OS), and disease-specific survival (DSS). The parameters of local failure, DM, and OS are as described in the initial reports (14–16). Because no relationship was observed between Ki-67 SI and local failure, the focus of this report was on the other end points. For DSS, failure was defined as death due to prostate cancer, protocol treatment toxicity, or unknown cause of death with active disease. DM was defined as clinical or radiological evidence of disease outside the pelvis. OS and DSS were measured from the date of randomization to the date of death or last follow-up date, if the patient did not fail. Time to DM was measured from the date of randomization to the date of failure, death, or the last follow-up date, if the patient did not fail.

Statistical Analysis. This analysis was performed using the 456 eligible and analyzable patients from RTOG Protocol 86-10. Of these, there were 108 patients who had KI-67 SI determinations available. As of June 30, 2000, the median follow-up in the study cohort was 6 years (range, 5 months to 11.8 years) for all patients and 9 years (range, 6–11.8 years) for living patients.

Based on Ki-67 SI expression, the data were dichotomized at the median value of 7.1% and at 3.5%; the latter was based on prior results (12). Analysis of the distribution of patients by Ki-67 SI and various potential prognostic factors was conducted by the Pearson χ^2 test with the Yates correction factor. Estimates of OS were derived using the Kaplan-Meier method (17). The cumulative incidence approach was used to estimate DM and DSS (18). This method adjusts for competing risks, such as dying without the recurrence of prostate cancer. Statistical comparisons of OS, DSS, and DM were made using the log-rank test (19).

Multivariate Cox proportional hazard models were applied to each of the end points (20). The initial multivariate analyses were restricted to patients for whom Ki-67 SI was available. The prognostic importance of Ki-67 SI was appraised after adjusting for treatment assignment, clinical stage, age (for the OS model), and Gleason score as fixed covariates (20). All factors were considered as dichotomous

Table 2 Univariate analyses of Ki-67 SI[a] data using the 7.1% cut point (n = 108)

End point	Ki-67 SI	N	Failures	5-year estimate	RR[b]	P[c]
Distant metastasis	≤7.1%	55	20	20.0%	2.36 (1.35–4.11)	0.0025
	>7.1%	53	34	56.8%		
Disease-specific survival	≤7.1%	55	10	92.8%	3.36 (1.62–6.98)	0.0011
	>7.1%	53	27	62.3%		
Overall survival	≤7.1%	55	37	70.9%	1.37 (0.87–2.15)	0.16
	>7.1%	53	40	49.0%		

[a] SI, staining index; RR, relative risk.
[b] A risk ratio of 1 indicates no difference between the two subgroups. The 95% confidence intervals for relative risk are shown in parentheses.
[c] χ^2 statistics.

variables and coded as follows: treatment assignment, 0 (radiotherapy alone) *versus* 1 (radiotherapy + AD); clinical stage, 0 (T_2) *versus* 1 (T_3); age, 0 (<75 years) *versus* 1 (≥75 years); grouped Gleason sums, 0 (sums of 2–6) *versus* 1 (sums of 7–10); p53, 0 (negative) *versus* 1 (positive); and Ki-67 SI, 0 (≤3.5%) *versus* 1 (>3.5%). In addition, a multivariate analysis was performed that considered an interaction between Ki-67 and treatment assignment for each end point. The fitted parameter from the Cox model is used to estimate the relative risk associated with each prognostic variable and corresponding 95% confidence interval. A ratio of 1 would indicate no difference between the two subgroups. The greater the difference is from 1, the greater the difference in the failure rates between the two subgroups. The treatment effect was modeled in such a way that a value < 1 favored the addition of hormones. Ki-67 SI was modeled in a way that a value > 1 indicates a greater risk of failure for patients with Ki-67 SI > 3.5%. All of the statistical comparisons were made with two-tailed tests.

A second type of multivariate analysis adjusted for two additional factors, p53 and missing tumor determinations. Of the 456 assessable patients, 108 (24%) had Ki-67 SI determinations, and 129 (28%) had p53 determinations. Both were available in 79 (17%) patients. There are potential analytical problems due to the missing values. Selection bias may occur, wherein the patients in whom the assays were done do not constitute a random sample from the whole study. As a consequence, the study cohort may have a better or worse outcome than the parent cohort. Moreover, when cases with missing values are excluded from the analysis, the number of patients to be analyzed may be relatively small, compromising the statistical power needed to detect clinically meaningful differences.

To adjust for the problem of missing values in the second multivariate analysis, two variables (instead of one) were used to evaluate each marker. For Ki-67 SI, patients were classed into three categories: determination not done; Ki-67 SI ≤ 3.5%; and Ki-67 SI > 3.5%. For p53, patients were classed into three categories: determination not done; negative; and positive. The first variable for Ki-67 SI would then be 0 (Ki-67 SI ≤ 3.5%/not done) *versus* 1 (Ki-67 SI > 3.5%), and the second variable would be 0 (Ki-67 SI > 3.5%/not done) *versus* 1 (Ki-67 SI ≤ 3.5%). The estimated relative risk of Ki-67 SI was figuratively obtained by subtracting out the two variables. The 27 patients without centrally reviewed Gleason scores were excluded, leaving 429 patients for the analysis.

RESULTS

The positive staining of Ki-67 was distinct, and the Ki-67 SI varied significantly from case to case. The Ki-67 SI ranged from 0.2% to 45.5%, with a median of 7.1% and a mean of 8.9%. Table 1 shows that 51% of cases had a Ki-67 SI of ≤7.1%, whereas 34% had a Ki-67 SI of ≤3.5%. By the χ^2 test, Ki-67 SI > 7.1% and Ki-67 SI > 3.5% were associated with higher combined Gleason score and younger age (borderline for the 3.5% cut point), but not with clinical T category or assigned treatment (Table 1). The focus of the univariate and multivariate analyses described was using the end points of DM, DSS, and OS. No relationship was found

Table 3 Univariate analyses of Ki-67 SI[a] data using the 3.5% cut point (n = 108)

End point	Ki-67 SI	N	Failures	5-year estimate	RR[b]	P[c]
Distant metastasis	≤3.5%	37	8	13.5%	3.77 (1.77–8.01)	0.0005
	>3.5%	71	46	50.8%		
Disease-specific survival	≤3.5%	37	4	97.3%	4.62 (1.63–13.07)	0.0039
	>3.5%	71	33	67.7%		
Overall survival	≤3.5%	37	22	70.2%	1.39 (0.85–2.3)	0.18
	>3.5%	71	55	54.9%		

[a] SI, staining index; RR, relative risk.
[b] A risk ratio of 1 indicates no difference between the two subgroups. The 95% confidence intervals for relative risk are shown in parentheses.
[c] χ^2 statistics.

between local failure and Ki-67 SI, and these data are not described.

Tables 2 and 3 display the univariate survival results. Both Ki-67 SI >7.1% and Ki-67 SI >3.5% were significantly associated with higher rates of DM and lower rates of DSS. However, Ki-67 SI was not significantly associated with OS. Based on the univariate results, the 3.5% cut point appears to be as good as the median value (7.1%) for the locally advanced study population here. Moreover, the 3.5% cut point is more relevant for the analysis of the typical contemporary patient with earlier disease (12). Because a major emphasis of this investigation was to validate the prior results showing that the Ki-67 SI 3.5% cut point is predictive of patient outcome, the remaining analyses center on this categorization. Fig. 1 displays the cumulative incidence curves demonstrating the effect of Ki-67 SI on DM and DSS. Also shown are the Kaplan-Meier curves for OS. No relationship was observed between Ki-67 SI status and the study randomization of EBRT *versus* AD + EBRT, although the statistical power to detect a difference is low.

Multivariate analyses restricted to patients with Ki-67 data showed that patients with a Ki-67 SI > 3.5% had a higher risk of DM and a higher risk of death due to prostate cancer, but Ki-67 SI > 3.5% was not associated with a higher risk of overall death (Table 4). No other factor was found to be associated with DM or DSS.

RTOG Protocol 86-10 included 456 assessable patients. Thus, there were 348 cases for whom Ki-67 SI data were missing. A separate set of analyses was performed to determine whether the subgroup analyzed for Ki-67 SI was representative of the patient cohort. Table 5 displays the distribution of pretreatment characteristics of patients by the Ki-67 SI subgroup and the remaining patients. No statistically significant differences were seen. However, patients with Ki-67 data had a borderline increased risk of DM and had significantly lower DSS and OS rates than those with missing Ki-67 data (Table 6).

In a previously reported analysis, p53 status was found to be a significant prognostic variable for survival, but it was only available for 79 patients with a Ki-67 determination. As a consequence, a multivariate analysis using Cox proportional hazards regression that adjusted for this population selection effect was done using 429 patients (see "Materials and Methods"). Table 7 shows that after adjusting for population effects and p53 status, in addition to Gleason score, clinical stage, and assigned treatment, Ki-67 was an independent prognostic factor for DM and DSS. Gleason score and p53 were associated with DM and DSS, whereas clinical stage and treatment regimen (EBRT alone *versus* AD + EBRT) were not. Patient age, clinical T category, Gleason score, and p53 were associated with OS.

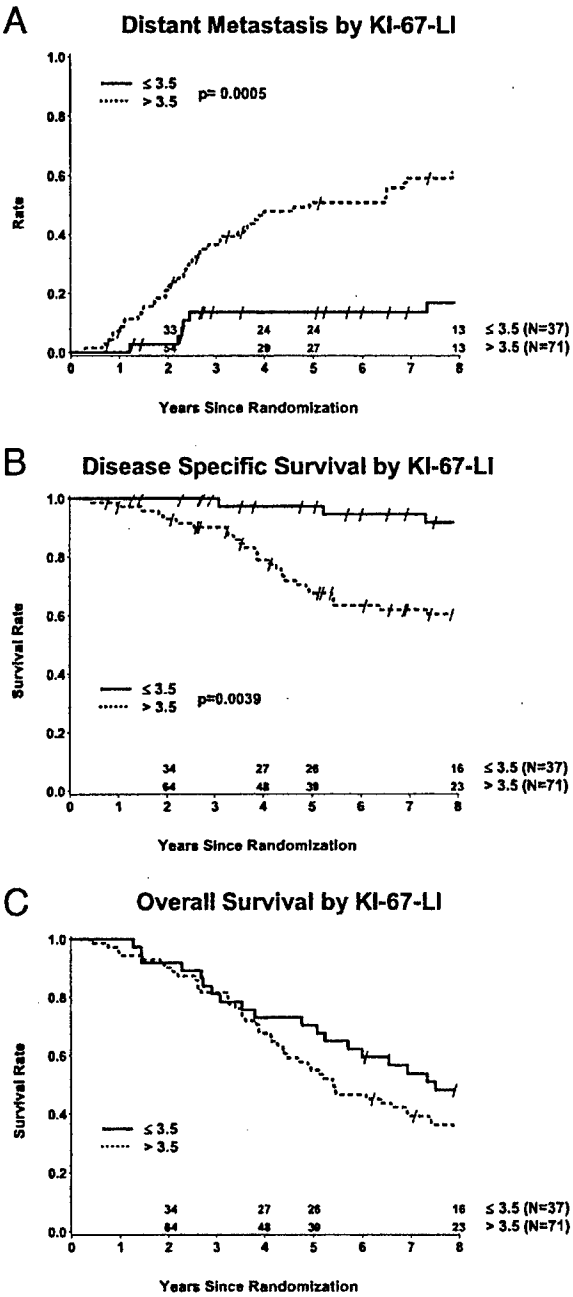

Fig. 1 Relationship of Ki-67 staining index at the 3.5% cut point to distant metastasis (*A*), disease-specific survival (*B*), and overall survival (*C*). The distant metastasis curves were generated using cumulative incidence estimates, and the disease-specific survival and overall survival curves were generated using Kaplan-Meier estimates.

DISCUSSION

Ki-67 expression has been related to patient outcome for a number of tumor types, including prostate cancer. Although there are several reports of the correlation of Ki-67 immunohistochemical staining status and prostate cancer progression, few have examined the predictive value of this biomarker in patients treated with radiotherapy (10–12). In addition, the investigation of Ki-67 SI as a potential predictor of prostate cancer patient outcome has previously been done in mainly small, single-institution studies. To our knowledge this is the first study of the expression of this marker in a well-defined group treated in a large multi-institutional randomized trial.

Table 4 Cox proportional hazards regression using only patients with Ki-67 SI[a] data (N = 108)

End point	Variable	Comparison	RR[b]	P[c]
DM	Stage	T_2 *versus* T_3	1.06 (0.57–1.94)	0.84
	Treatment	RT alone *versus* RT+AD	0.62 (0.34–1.12)	0.11
	Ki-67 SI	≤3.5 *versus* >3.5	3.41 (1.48–7.84)	0.0038
	Gleason	2–6 *versus* 7–10	1.62 (0.75–3.52)	0.21
DSS	Stage	T_2 *versus* T_3	1.13 (0.53–2.40)	0.74
	Treatment	RT alone *versus* RT+AD	0.99 (0.49–2.00)	0.99
	Ki-67 SI	≤3.5 *versus* >3.5	4.24 (1.43–12.59)	0.009
	Gleason	2–6 *versus* 7–10	1.17 (0.47–2.92)	0.73
OS	Stage	T_2 *versus* T_3	1.13 (0.66–1.91)	0.64
	Treatment	RT alone *versus* RT+AD	0.86 (0.53–1.41)	0.56
	Ki-67 SI	≤3.5 *versus* >3.5	1.36 (0.78–2.39)	0.27
	Gleason	2–6 *versus* 7–10	1.05 (0.59–1.87)	0.85
	Age (yrs)	<75 *versus* ≥75	1.06 (0.63–1.80)	0.80

[a] SI, staining index; RR, relative risk; DM, distant metastasis; DSS, disease-specific survival; RT, radiotherapy; AD, androgen deprivation; OS, overall survival.

[b] A risk ratio of 1 indicates no difference between the two subgroups. The 95% confidence intervals for relative risk are shown in parentheses.

[c] Cox proportional hazards model.

As such, there is knowledge of the patient characteristics and outcome of those in whom the marker was not available. Table 6 shows that there were differences in DSS and OS between the patients in whom Ki-67 SI was determined and those in whom it was missing. The cause of this difference is unclear. It is a rare biomarker study in which the features and outcome of those excluded are known and the potential selection bias is acknowledged. Tissue might not have been available for Ki-67 SI analysis because the original archival material was not accessible or because there was insufficient tumor tissue in the retrieved blocks. As has been described previously (15), it is possible to adjust for the selection bias by inclusion of the patients with missing Ki-67 SI data in multivariate analysis. When this was done (Table 7), Ki-67 SI still significantly predicted for DM and DSS.

One of the inherent problems with immunohistochemical analyses is the wide variation in cut points used to define associations with patient outcome. This consideration is particularly apropos for Ki-67 SI analyses. As we have pointed out previously (12), a number of Ki-67 SI cut points (1–25%) have been found to be predictive of freedom from biochemical or clinical failure in men with prostate cancer. These cut points largely were chosen around the median Ki-67 SI for the cohort studied. The differences in median Ki-67 SI are probably related primarily to the aggressiveness of the tumors but also could be influenced by staining technique. A critical step is antigen retrieval, which could be affected by the original fixation parameters before embedding in paraffin, length of heating, and method of heating.

There are several unique features of the current Ki-67 SI study. First, the tissue was collected from around the country in the setting of a multi-institutional randomized trial. The handling and fixation of the biopsy material were not controlled and therefore reflect current practices in the United States. Second, the staining was performed in an autostainer in three batches. Positive controls for the batches showed identical proportions of proliferating cells. Third, the cohort studied was treated rather uniformly with EBRT ± AD on protocol using conventional techniques. For these reasons, the associations described should be generally applicable in the community.

The other important aspect of the RTOG Protocol 86-10 population is that it was composed of cases with very high-risk characteristics. Consequently, the median Ki-67 SI was 7.1%, which is substantially greater than the 2.3% observed in the patients with favorable- to intermediate-risk features comprising the cohort from M. D. Anderson Cancer Center (MDACC) used in our prior analysis (12). In the prior analysis, Ki-67 SI was a significant predictor of biochemical failure in both univariate and multivariate Cox proportional hazards regression analyses. Those with a Ki-67 SI of >3.5% were identified as having a particularly poor prognosis, with a freedom from biochemical failure rate of 33%, *versus* a rate

Table 5 Characteristics of patients in RTOG[a] Protocol 86-10 with determined or missing Ki-67 data (N = 429)

Characteristics	Determined Ki-67 data (N = 108)	Missing Ki-67 data (N = 348)	P[b]
Age (yrs)			
<75	79 (73%)	246 (71%)	0.62
≥75	29 (27%)	102 (29%)	
Combined Gleason score			
Unknown	1 (<1%)	26 (7%)	
Determined	107 (99%)	322 (93%)	
2–6	27 (25%)	102 (32%)	0.20
7–10	80 (75%)	220 (68%)	
Clinical stage			
T_2	31 (28%)	106 (30%)	0.72
T_3	77 (72%)	242 (70%)	
Assigned treatment			
RT alone	60 (56%)	170 (49%)	0.22
RT + HT	48 (44%)	178 (51%)	

[a] RTOG, Radiation Therapy Oncology Group; RT, radiotherapy; HT, hormone therapy.

[b] χ^2 statistics.

Table 6 Univariate analyses of missing *versus* determined Ki-67 data (N = 429)

End point	Ki-67	N	Failures	RR[a]	P[b]
Distant metastasis	Determined	108	54	1.3 (0.95–1.8)	0.09
	Missing	348	135		
Disease-specific survival	Determined	108	37	1.54 (1.0–2.2)	0.027
	Missing	348	82		
Overall survival	Determined	108	77	1.43 (1.1–1.8)	0.0075
	Missing	348	182		

[a] RR, relative risk. A risk ratio of 1 indicates no difference between the two subgroups. The 95% confidence intervals for relative risk are shown in parentheses.
[b] Cox proportional hazards model.

of 76% for those with a Ki-67 SI of ≤3.5%. Of the patients investigated by prostate biopsy for rising prostate-specific antigen (PSA), 65% had documented local persistence of disease. The number of patients with DM in the MDACC study was too small (n = 4) to assess for any correlation with Ki-67 SI.

Despite the inherent differences in the MDACC (12) and RTOG Protocol 86-10 cohorts, there were some parallels in the relationships found. Whereas biochemical failure was not investigated in the RTOG group because this was a pre-PSA era study, Ki-67 SI was significantly associated with DM and DSS. The strong association of Ki-67 at the 3.5% cut point to biochemical failure (relative risk = 2.8) in the MDACC series was likewise robust for the DM and DSS end points (relative risk = 3.41 and 4.24, respectively) in the current study. Thus, although there were pronounced differences in the characteristics of the MDACC and RTOG patient populations, the 3.5% Ki-67 SI cut point was validated. We also found that the median value of 7.1% in the RTOG Protocol 86-10 group was predictive of DM and DSS.

Another finding in the current analysis was that there was no association between Ki-67 SI and local failure. Some possible reasons include the following: (*a*) Ki-67 SI is a much stronger determinant of DM than it is a predictor of radiation response; (*b*) the RTOG Protocol 86-10 patient population was very advanced, and in the setting of DM, the documentation of local disease was probably underdocumented; and (*c*) in the absence of PSA as an early indication of treatment failure in this pre-PSA study, the impetus for prostate biopsies in the absence of obvious clinical local progression would not have been present. Further investigation of Ki-67 SI in patients treated with radiotherapy in the PSA era would resolve these questions.

Also examined herein was the association of Ki-67 SI to OS. Despite the strong relationship of a high Ki-67 SI to the development of DM and death due to prostate cancer, no relation to OS was seen. The data suggest that this may be due to dilution of disease-specific deaths by deaths from intercurrent causes.

In summary, Ki-67 SI at the 3.5% cut point was validated as a significant correlate of outcome for prostate cancer patients treated with EBRT or AD + EBRT. The relationship to DM and DSS described appeared to be independent of whether AD was given and was independent of p53 immunohistochemical staining status. The main drawback of the study is that only 108 cases were available. Further testing in a larger cohort should be undertaken before incorporating Ki-67 SI in future clinical trials.

Table 7 Multivariate analyses (all patients;[a] inclusion of P53 in model; N = 429)

End point	Variable	Comparison	RR[b,c]	P[d]
Distant metastasis	Stage	T_2 *versus* T_3	1.28 (0.92–1.79)	0.13
	Treatment	RT alone *versus* RT+HT	0.77 (0.57–1.03)	0.08
	Ki-67 SI	≤3.5 *versus* >3.5	3.31 (1.48–7.39)	0.0034
	Gleason	2–6 *versus* 7–10	2.05 (1.40–3.0)	0.0002
	P53	No *versus* yes	2.16 (1.19–3.93)	0.01
Disease-specific survival	Stage	T_2 *versus* T_3	1.32 (0.87–2.02)	0.18
	Treatment	RT alone *versus* RT+HT	0.76 (0.52–1.10)	0.15
	Ki-67 SI	≤3.5 *versus* >3.5	3.19 (1.12–9.09)	0.02
	Gleason	2–6 *versus* 7–10	3.39 (1.93–5.97)	<0.0001
	p53	No *versus* yes	2.37 (1.21–4.64)	0.01
Overall survival	Stage	T_2 *versus* T_3	1.40 (1.05–1.86)	0.01
	Treatment	RT alone *versus* RT+HT	0.87 (0.68–1.12)	0.30
	Ki-67 SI	≤3.5 *versus* >3.5	1.34 (0.80–2.25)	0.25
	Gleason	2–6 *versus* 7–10	1.46 (1.09–1.96)	0.009
	Age (yrs)	<75 *versus* ≥75	1.48 (1.12–1.94)	0.004
	p53	No *versus* yes	1.92 (1.11–3.29)	0.01

[a] All patients with all factors present, allowing cases with missing Ki-67 values.
[b] RR, relative risk; RT, radiotherapy; HT, hormone therapy
[c] A risk ratio of 1 indicates no difference between the two groups. The 95% confidence intervals for relative risk are shown in parentheses.
[d] Cox proportional hazards model.

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ORIGINAL REPORT

Ki-67 Staining Is a Strong Predictor of Distant Metastasis and Mortality for Men With Prostate Cancer Treated With Radiotherapy Plus Androgen Deprivation: Radiation Therapy Oncology Group Trial 92-02

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ABSTRACT

Purpose
The Ki-67 staining index (Ki67-SI) has been associated with prostate cancer patient outcome; however, few studies have involved radiotherapy (RT) -treated patients. The association of Ki67-SI to local failure (LF), biochemical failure (BF), distant metastasis (DM), cause-specific death (CSD) and overall death (OD) was determined in men randomly assigned to short term androgen deprivation (STAD) + RT or long-term androgen deprivation (LTAD) + RT.

Patients and Methods
There were 537 patients (35.5%) on Radiation Therapy Oncology Group (RTOG) 92-02 who had sufficient tissue for Ki67-SI analysis. Median follow-up was 96.3 months. Ki67-SI cut points of 3.5% and 7.1% were previously found to be related to patient outcome and were examined here in a Cox proportional hazards multivariate analysis (MVA). Ki67-SI was also tested as a continuous variable. Covariates were dichotomized in accordance with stratification and randomization criteria.

Results
Median Ki67-SI was 6.5% (range, 0% to 58.2%). There was no difference in the distribution of patients in the Ki-67 analysis cohort (n = 537) and the other patients in RTOG 92-02 (n = 977) by any of the covariates or end points tested. In MVAs, Ki67-SI (continuous) was associated with LF ($P = .08$), BF ($P = .0445$), DM ($P < .0001$), CSD ($P < .0001$), and OD ($P = .0094$). When categoric variables were used in MVAs, the 3.5% Ki67-SI cut point was not significant. The 7.1% cut point was related to BF ($P = .09$), DM ($P = .0008$), and CSD ($P = .017$). Ki67-SI was the most significant correlate of DM and CSD. A detailed analysis of the hazard rates for DM in all possible covariate combinations revealed subgroups of patients treated with STAD + RT that did not require LTAD.

Conclusion
Ki67-SI was the most significant determinant of DM and CSD and was also associated with OD. The Ki67-SI should be considered for the stratification of patients in future trials.

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INTRODUCTION

The quantification of the expression of the Ki-67 antigen using immunohistochemical methods has been shown to provide an estimate of the growth fraction.[1-4] As typically slow-growing tumors, most prostate cancers treated with radiotherapy have been found to have relatively low Ki-67 staining indices (Ki67-SIs).[5-7] However, there is a wide spectrum of values, with some cases having a Ki67-SI of more than 50% and others having no discernable activity. Moreover, Ki67-SI has consistently been a significant correlate of outcome for prostate cancer patients treated definitively with radical prostatectomy or radiotherapy (RT).[5-15] The relationship of Ki67-SI to outcome is a continuous function; the higher the value, the greater the risk of biochemical or clinical failure.

There are several reasons the Ki67-SI has not been adopted in clinical practice or

clinical trials of prostate cancer patients. First, prior studies have involved relatively small numbers of patients. In terms of radiotherapy as primary treatment, there are also few studies to draw meaningful conclusions.[5-7,14] Second, there is little published on the relationship of Ki67-SI to outcome after the combination of RT plus short term androgen deprivation (STAD),[16] and no data are available on the relationship of Ki67-SI to outcome after RT plus long-term androgen deprivation (LTAD). Third, there is no consistently defined cut point that has been identified.

In the analysis described here, archival diagnostic tissue samples from a large cohort of patients randomly assigned to receive either STAD + RT or LTAD + RT in Radiation Therapy Oncology Group (RTOG) protocol 92-02 were stained for Ki-67 using MIB-1 antibody. This analysis of Ki67-SI is one of the largest biomarker studies ever performed in patients with prostate cancer. The results lend credibility to the premise that Ki67-SI is a valuable independent correlate of prostate cancer patient outcome that should receive broader consideration in the pretreatment evaluative process, along with Gleason score, initial pretreatment prostate-specific antigen (iPSA) level, and T stage.

Patients and Methods

Patient Characteristics

There were 1,514 analyzable patients in RTOG protocol 92-02,[17] with 761 in the STAD + RT arm and 753 in the LTAD + RT arm. Tissue was available for Ki67-SI analysis in 537 patients (35.4%), with 257 randomly assigned to the STAD + RT arm and 280 to the LTAD + RT arm. For the study cohort, median age was 70 years (range, 43 to 88 years), and median iPSA was 20.2 ng/mL (range, 0.1 to 295.0 ng/mL). There were 359 patients (67%) with an iPSA of $\leq$ 30 ng/mL and 237 patients (44%) with T2c disease. An institutional Gleason score was available in four patients, with scores of 2 to 6 in 192 patients (39%). Median follow-up for the parent cohort (n = 1,514) was 101.1 months, and for the Ki67-SI available test cohort (n = 537) was 96.3 months.

Treatment Characteristics

As described previously,[17] STAD was begun 2 months before RT and was continued during RT. Total androgen deprivation was accomplished with flutamide at 750 mg/d plus an LHRH agonist. The initial first 4 months of LTAD were structured identically to the first 4 months of STAD, and then an additional 24 months of LHRH agonist was given after the completion of RT. The prescription RT dose was 65 to 70 Gy in both arms. The whole pelvis was treated to 44 to 46 Gy, followed by an additional 20 to 26 Gy to the prostate. The median RT dose was 68.4 Gy.

Ki-67 Staining

The staining of Ki-67 using MIB-1 antibody has been described previously.[6,16] Briefly, paraffin-embedded archival sections (4 microns) were deparaffinized in xylene and then rehydrated in graded ethyl alcohol. Antigen retrieval was accomplished by heating the sections in 10 mmol/L citrate buffer pH 6.0 for 50 minutes using a pressure cooker (BioCare Medical, Walnut Creek, CA). Hydrogen peroxide at 0.3% was administered to the slides for 5 minutes. The slides were then incubated with the monoclonal antibody MIB-1 (DAKO Corp, Carpinteria, CA) for 10 minutes. Biotinylated secondary antibody was applied for 10 minutes, followed by incubation with streptavidin peroxidase (DAKO LSAB2, k0675) for 10 minutes. The slides were rinsed and stained with diaminobenzidine chromogen solution (ResGen Invitrogen Corp, Carlsbad, CA) and counterstained with routine hematoxylin. Staining was accomplished using a DAKO Autostainer (DAKO). Negative staining controls consisted of slides stained with omission of the primary antibody. Normal tonsil sections, which had previously been studied by flow cytometry to determine the percentage of proliferating cells, were used as positive controls.

End Points

The failure event for overall death (OD) was defined as death due to any cause. The following was considered a failure event in assessing cause-specific death (CSD): death certified as due to prostate cancer, death due to complications of treatment, death from unknown causes with active malignancy (clinical disease relapse), or from another cancer with documented bone metastases attributed to prostate cancer before the appearance of the second independent cancer. Local failure (LF) was assessed by palpation and defined as an increase in tumor volume of 25% or local persistence of palpable tumor beyond 18 months. Distant metastasis (DM) was defined as radiographic or clinical evidence of hematogenous spread. The definition of biochemical failure (BF) was modeled after the American Society of Therapeutic Radiology and Oncology consensus definition.[18] BF was defined as three consecutive rises or the institution of hormone treatment for a rising PSA or a post-treatment PSA nadir level > 4.0 ng/mL. All time events, with the exception of BF, were measured from the date of randomization to the date of their occurrence or last follow-up. BF was measured from the date of randomization to the midpoint date between the postirradiation date of nadir PSA and the date of the first of the three consecutive rises.

The Ki67-SI was defined as the percentage of tumor cells that displayed nuclear MIB-1 staining. Whenever possible, 2,000 tumor cells were counted using an eyepiece grid.

Statistics

All pretreatment characteristics were dichotomized. Age was dichotomized by the median age in the entire cohort. PSA ($\leq$ 30 ng/mL v > 30 ng/mL) and T stage (T2c v T3 or T4) were dichotomized by the stratification groupings in RTOG protocol 92-02, and Gleason score was dichotomized as 2 to 6 versus 7 to 10. Statistical comparisons to assess whether missing Ki-67 data were dependent on pretreatment characteristics, assigned treatment, or outcome were carried out using χ^2 tests and Cox proportional hazards models.[19] Ki67-SI was dichotomized for the primary analyses as either $\leq$ 3.5 versus > 3.5[6] or $\leq$ 7.1 versus > 7.1.[16] Additional Ki67-SI groupings were also explored. Cox proportional hazards models were utilized to identify the relationship of Ki67-SI to OD, CSD, DM, LF, and BF. Actuarial estimates of OD and CSD rates were calculated using the Kaplan-Meier method.[20] The cumulative incidence method[21] was used to estimate LF, DM and BF rates.

Results

The Ki67-SI was determined in 537 of 1,514 patients in the parent cohort. Analyses were performed to determine if the

Table 1. Characteristics of Patients With Ki67-SI Determined Versus Missing (N = 1,514)

Characteristic	Determined Ki-67 Data (n = 537)		Missing Ki-67 Data (n = 977)		P*
	No. of Patients	%	No. of Patients	%	
Age, years					
< 70	242	45.1	433	44.3	.78
≥ 70	295	54.9	544	55.7	
Gleason score					
Unknown	41	7.6	61	6.3	
Determined	496	92.4	916	93.7	
2-6	192	38.7	385	42.0	.23
7-10	304	61.3	531	58.0	
PSA, ng/mL					
≤ 30	359	66.9	652	66.7	.96
> 30	178	33.1	325	33.3	
Clinical stage					
T2c	237	44.1	449	46.0	.50
T3-4	300	55.9	528	54.0	
Assigned treatment					
LTAD + RT	280	52.1	473	48.4	.17
STAD + RT	257	47.9	504	51.6	

Abbreviations: SI, staining index; PSA, prostate-specific antigen; LTAD, long-term androgen deprivation; RT, radiotherapy; STAD, short-term androgen deprivation.
*χ^2 statistics.
†There were 642 patients randomly assigned and eligible with tissue available.

Ki67-SI test cohort was representative of the 977 cases in which Ki67-SI was not available. Table 1 shows that there was no statistically significant difference in pretreatment characteristics between the test cohort and the remaining cases in the parent cohort. Table 2 displays univariate comparisons of the test cohort to the Ki67-SI absent group in terms of the end points of OD, CSD, DM, LF and BF. No significant differences between the groups were identified.

Median Ki67-SI was 6.5, with a range of 0 to 58.2. Based on prior analyses of prostate cancer patients treated with RT ± STAD at The University of Texas M.D. Anderson Cancer Center[6] and in RTOG trial 86-10,[16] Ki67-SI was dichotomized at ≤ 3.5 (n = 121) versus > 3.5% (n = 416) and ≤ 7.1 (n = 290) versus > 7.1 (n = 247); these cut points have previously been shown to be predictive of outcome. Because the 3.5% cut point was not found to be significantly related to any of the end points listed in Table 2, the discussion to follow focuses on the 7.1% cut point.

As shown in Table 3, pretreatment characteristics and assigned treatment were generally well distributed between those who had a Ki67-SI ≤ 7.1 or > 7.1%, with the exception of institutional Gleason score. There was a greater

Table 2. Univariate Analyses of Patient Outcome for Those With Ki67-SI Determined Versus Missing (N = 1,514)

End Point	Ki-67	No. of Patients	Treatment Failures	Risk*		P†
				RR	95% CI	
Overall death	Determined	537	159	1.01	0.83 to 1.23	.92
	Missing	977	289			
Cause-specific death	Determined	537	54	1.12	0.80 to 1.57	.51
	Missing	977	88			
Distant metastasis	Determined	537	76	0.96	0.73 to 1.26	.76
	Missing	977	147			
Local failure	Determined	537	54	1.04	0.75 to 1.46	.80
	Missing	977	91			
Biochemical failure‡	Determined	533	194	0.99	0.83 to 1.18	.90
	Missing	969	348			

Abbreviations: SI, staining index; RR, relative risk.
*A risk ratio of 1 indicates no difference between the two subgroups. The Ki-67 indicator was coded as 0: missing, 1: determined.
†χ^2 statistics.
‡Twelve patients (four with determined Ki-67 and eight missing Ki-67) have unknown biochemical progression status.

Table 3. Distribution of Patients With Ki67-SI Data (n = 537) by the 7.1% Cut Point

Characteristics	≤ 7.1 (n = 290)		> 7.1 (n = 247)		P*
	No. of Patients	%	No. of Patients	%	
Age, years					
< 70	132	45.5	110	44.5	.82
≥ 70	158	54.5	137	55.5	
Gleason score					
2-6	126	46.7	66	29.2	.0001
7-10	144	53.3	160	70.8	
PSA, ng/mL					
≤ 30	195	67.2	164	66.4	.84
> 30	95	32.8	83	33.6	
T stage					
T2c	128	44.1	109	44.1	.99
T3-4	162	55.9	138	55.9	
Assigned treatment					
LTAD + RT	145	50.0	135	54.7	.28
STAD + RT	145	50.0	112	45.3	

Abbreviations: SI, staining index; PSA, prostate-specific antigen; LTAD, long-term androgen deprivation; RT, radiotherapy; STAD, short-term androgen deprivation.
*χ^2 statistics.

percentage of Gleason score 7 to 10 when the Ki67-SI was > 7.1% (P = .0001). In univariate analyses (Table 4) Ki67-SI > 7.1% was significantly related to CSD (P = .0053), and DM (P = .0003; also shown in Fig 1); although borderline significant associations were noted for BF (P = .0504) and OD (P = .0551). Table 5 displays the multivariate analyses, demonstrating that dichotomized Ki67-SI at the 7.1% cut point is an independent, significant correlate of DM and CSD. A borderline relationship was seen between Ki67-SI and BF.

The most clinically important, as well as the most significant, association was between Ki67-SI and progression to DM. The data validate that when the Ki67-SI is > 7.1% there is a substantially increased risk of distant metastasis; however, the relationship is a continuous function. The results in Table 6 illustrate how the relative risk of distant metastasis increases as the Ki67-SI cut point increases up to between 9% and 10%. The optimal cut point for the patient population participating in RTOG protocol 92-02 seems to be in this range. In support of these findings, Table 7 shows that the significance of Ki67-SI in the multivariate analyses was strengthened when it was used as a continuous variable. For this analysis iPSA was also included as a continuous variable, as the relationships for iPSA as a continuous variable were stronger as well. As a continuous variable, Ki67-SI was significantly associated with all of the end points tested, except for LF.

Table 4. Univariate Analyses of the Ki67-SI Test Cohort (n = 537) Using the 7.1% Cut Point

End Point	Ki-67	No. of Patients	Treatment Failures	% 5 Years	RR*	95% CI†	P‡
OD*	≤ 7.1	290	73	16.1			
	> 7.1	247	86	24.2	1.36	0.99 to 1.86	.0551
CSD*	≤ 7.1	290	18	3.3			
	> 7.1	247	36	11.7	2.25	1.27 to 3.98	.0053
DM	≤ 7.1	290	26	8.6			
	> 7.1	247	50	17.3	2.38	1.48 to 3.83	.0003
LF	≤ 7.1	290	26	8.2			
	> 7.1	247	28	10.4	1.32	0.78 to 2.26	.30
BF§	≤ 7.1	289	93	33.2			
	> 7.1	244	101	42.3	1.32	1.00 to 1.76	.0504

Abbreviations: RR, relative risk; OD, overall death; CSD, cause-specific death; DM, distant metastasis; LF, local failure; BF, biochemical failure.
*Actuarial estimates calculated using Kaplan-Meier methods. The cumulative incidence method was used to estimate LF, DM, and BF rates.
†Relative Risk: A risk ratio of 1 indicates no difference between the two subgroups. The Ki-67 variable was coded as 0: ≤ 7.1, 1: > 7.1. The 95% confidence interval is shown in parentheses.
‡χ^2 statistics.
§Four patients (one patient with Ki-67 ≤ 7.1 and three patients with Ki-67 > 7.1) have unknown biochemical progression status.

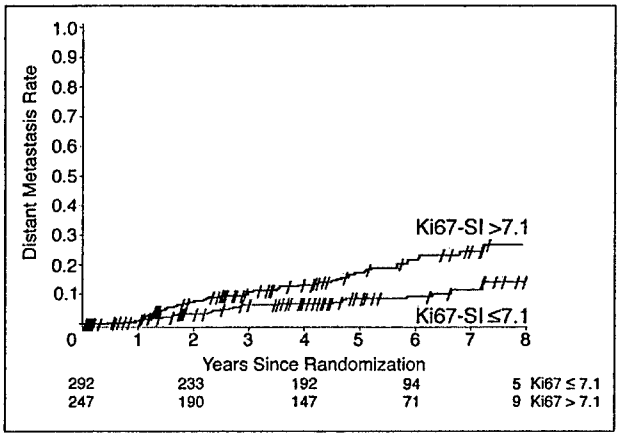

Fig 1. Cumulative incidence distant metastasis curves for Ki67-SI subdivided at the 7.1% cut point. SI, staining index. The numbers at risk at 0, 2, 4, and 6 years are shown.

Another analysis was performed to determine if Ki67-SI was useful in defining which patients in RTOG protocol 92-02 who were treated with STAD + RT would not require LTAD + RT. This was accomplished by testing the relationship of all possible prognostic subgroups (n = 16) based on the dichotomized variables of Gleason score (2 to 6 *v* 7 to 10), iPSA (≤ 30 *v* > 30 ng/mL), T stage (T2c *v* T3-T4) and Ki67-SI ≤ 7.1% *v* > 7.1%. Table 8 displays the estimated (or predicted) relative risk and CIs of distant metastasis from the Cox model for the 16 possible groups for patients randomly assigned to receive STAD + RT. To compensate for the multiple CIs, a Bonferroni correction was used to establish a new α level. The new α level was computed with the formula: α (adjusted) = α (old)/n, where the new α level is the probability that must be achieved to be significant given the number of comparisons, the old α is the level that formerly defined significance, and n is the number of comparisons (ie, CIs) reported. Thus, with an original α level of 0.05 and 16 comparisons, the new α level is 0.003, which translates to a 99.7% CI.

The reference group used for comparison was comprised of the most favorable patients randomly assigned to the LTAD + RT arm with an iPSA ≤ 30 ng/mL, Gleason score 2 to 6, T stage T2c, and Ki67-SI ≤ 7.1. All CIs constructed were compared to the reference group. There were four prognostic groups of patients on the STAD + RT arm that had a similar risk of developing distant metastasis as

Table 5. Cox Proportional Hazards Analysis of Ki-67 Using the 7.1% Cut Point (n = 496)

End Point	Variable	Group	RR	95% CI	P*
OD	Age, years	≥ 70	1.56	1.10 to 2.19	.0114
	Gleason Score	7-10	1.12	0.79 to 1.59	.52
	iPSA, ng/ml	> 30	0.84	0.59 to 1.19	.33
	T stage	T3-4	0.87	0.63 to 1.21	.41
	Treatment	STAD + RT	1.02	0.74 to 1.41	.91
	Ki67-SI	> 7.1	1.25	0.90 to 1.75	.19
CSD	Gleason score	7-10	1.01	0.55 to 1.87	.96
	iPSA, ng/ml	> 30	0.93	0.51 to 1.69	.81
	T Stage	T3-4	1.71	0.93 to 3.12	.08
	Treatment	STAD + RT	1.99	1.11 to 3.56	.0211
	Ki67-SI	> 7.1	2.07	1.14 to 3.76	.0174
DM	Gleason score	7-10	1.33	0.77 to 2.30	.30
	iPSA, ng/ml	> 30	1.26	0.77 to 2.05	.36
	T stage	T3-4	1.80	1.07 to 3.02	.0260
	Treatment	STAD + RT	1.86	1.13 to 3.04	.0139
	Ki67-SI	> 7.1	2.39	1.43 to 3.97	.0008
LF	Gleason score	7-10	1.12	0.62 to 2.04	.70
	iPSA, ng/ml	> 30	0.88	0.48 to 1.60	.67
	T stage	T3-4	1.18	0.67 to 2.08	.57
	Treatment	STAD + RT	2.33	1.29 to 4.19	.0048
	Ki67-SI	> 7.1	1.30	0.74 to 2.30	.36
BF	Gleason score	7-10	1.17	0.85 to 1.61	.34
	iPSA, ng/ml	> 30	1.62	1.20 to 2.19	.0017
	T stage	T3-4	1.28	0.94 to 1.74	.11
	Treatment	STAD + RT	3.69	2.67 to 5.09	< .0001
	Ki67-SI	> 7.1	1.30	0.96 to 1.76	.09

NOTE. The Ki67-SI variable was coded as 0: ≤ 7.1 and 1: > 7.1.
Abbreviations: RR, relative risk; OD, overall death; iPSA, initial pretreatment prostate-specific antigen; STAD + RT, short-term androgen deprivation with radiotherapy; SI, staining index; CSD, cause-specific death; DM, distant metastasis; LF, local failure; BF, biochemical failure.
*Cox proportional hazards regression statistic.

Table 6. Effect of Increasing Ki67-SI Cut Point on the Relative Risk of Distant Metastasis

	Relative Risk of Distant Metastasis*						
Cut point	≤ 3.5 (n = 121) v > 3.5 (n = 416)	≤ 7.1 (n = 290) v > 7.1 (n = 247)	≤ 8.0 (n = 326) v > 8.0 (n = 211)	≤ 9.0 (n = 354) v > 9.0 (n = 183)	≤ 10 (n = 377) v > 10 (n = 160)	≤ 11.0 (n = 398) v > 11.0 (n = 139)	≤ 12.7 (n = 428) v > 12.7 (n = 109)
Unadjusted	1.83	2.19	2.82	3.49	3.80	3.27	4.03
95% CI	0.84 to 3.57	1.36 to 3.54	1.77 to 4.50	2.19 to 5.55	2.40 to 6.02	2.08 to 5.15	2.56 to 6.35
Adjusted†	1.65	2.18	2.98	3.56	3.82	3.13	3.97
95% CI	0.81 to 3.37	1.31 to 3.64	1.81 to 4.91	2.17 to 5.85	2.34 to 6.24	1.92 to 5.08	2.44 to 6.46

Abbreviation: SI, staining index.
*A risk ratio of 1 indicates no difference between the two subgroups. An interval that does not contain 1 indicates a statistically significant difference between the subgroups at the 0.05 level.
†Relative risk of Ki-67 adjusted for Gleason score (institutional), PSA, T stage, and assigned treatment.

those in the LTAD + RT reference group. The four favorable STAD + RT groups were: (1) PSA ≤ 30 ng/mL, Gleason score 2 to 6, stage T2c, and Ki67-SI ≤ 7.1%; (2) PSA > 30, Gleason score 2 to 6, stage T2c, and Ki67-SI ≤ 7.1%; (3) iPSA ≤ 30 ng/mL, Gleason score 7 to 10, stage T2c, and Ki67-SI ≤ 7.1%, and (4) iPSA > 30 ng/mL, Gleason score 7 to 10, stage T2c, and Ki67-SI ≤ 7.1%). When the STAD + RT patients in these four relatively favorable groups were pooled (n = 59) and compared to LTAD + RT patients with the same prognostic features (n = 63), the 5-year distant metastasis rates were not different (5.2% *v* 3.6%; $P = .69$; Gray's test). The 5-year metastasis rates for the pooled unfavorable STAD + RT (n = 173) and the LTAD + RT (n = 201) were statistically different at 19.2% and 10.7% ($P = .01$; Gray's test), respectively. There seems to be no statistically significant difference in the inci-

Table 7. Cox Proportional Hazards Multivariate Analyses Using Ki67-SI and iPSA As Continuous Variables (n = 496)

End Point	Variable	Group	RR	95% CI*	P*
OD	Age, years	≥ 70	1.55	1.10 to 2.18	.0128
	Gleason score	7-10	1.05	0.74 to 1.49	.77
	iPSA, ng/ml	Increasing	1.00	0.91 to 1.09	.98
	T stage	T3-4	0.86	0.62 to 1.20	.38
	Treatment	STAD + RT	1.03	0.74 to 1.43	.86
	Ki67-SI	Increasing	1.73	1.14 to 2.63	.0094
CSD	Gleason score	7-10	0.90	0.48 to 1.68	.73
	iPSA, ng/ml	Increasing	1.03	0.90 to 1.18	.65
	T stage	T3-4	1.55	0.84 to 2.85	.16
	Treatment	STAD + RT	2.09	1.15 to 3.79	.0153
	Ki67-SI	Increasing	3.64	2.15 to 6.16	< .0001
DM	Gleason score	7-10	1.12	0.64 to 1.97	.69
	iPSA, ng/ml	Increasing	1.19	1.09 to 1.31	.0002
	T stage	T3-4	1.48	0.87 to 2.51	.15
	Treatment	STAD + RT	1.74	1.05 to 2.88	.0309
	Ki67-SI	Increasing	3.33	2.11 to 5.25	< .0001
LF	Gleason score	7-10	1.02	0.56 to 1.86	.95
	iPSA, ng/ml	Increasing	1.03	0.88 to 1.21	.71
	T Stage	T3-4	1.11	0.63 to 1.97	.72
	Treatment	STAD + RT	2.28	1.26 to 4.13	.0065
	Ki-67	Increasing	1.87	0.92 to 3.82	.0845
BF	Gleason score	7-10	1.13	0.82 to 1.56	.46
	iPSA, ng/ml	Increasing	1.14	1.07 to 1.22	.0001
	T stage	T3-4	1.21	0.89 to 1.64	.24
	Treatment	STAD + RT	3.60	2.61 to 4.98	< .0001
	Ki67-SI	Increasing	1.50	1.01 to 2.23	.0445

Abbreviations: iPSA, initial pretreatment prostate-specific antigen; RR, relative risk; OD, overall death; STAD, short-term androgen deprivation; RT, radiotherapy; CSD, cause specific death; SI, staining index; DM, distant metastasis; LF, local failure; BF, biochemical failure.
*Cox proportional hazards regression statistic.

Table 8. Prognostic Groups for Distant Metastasis Based on iPSA, Gleason Score, T Stage, and Ki67-SI

	Treatment	iPSA	Gleason Score	T Stage	Ki67-SI	RR*	99.7% CI	% 5-Year DM†
Reference Group (n = 25)	LTAD + RT	≤ 30	2-6	T2c	≤ 7.1	1.00		3.4
Prognostic Group (n = 236)								
1 (n = 28)	STAD + RT	≤ 30	2-6	T2c	≤ 7.1	1.86	0.88 to 3.91	6.2
2 (n = 9)	STAD + RT	> 30	2-6	T2c	≤ 7.1	2.33	0.85 to 6.42	7.7
3 (n = 17)	STAD + RT	≤ 30	7-10	T2c	≤ 7.1	2.47	0.82 to 7.48	8.2
4 (n = 9)	STAD + RT	> 30	7-10	T2c	≤ 7.1	3.11	0.91 to 10.63	10.2
5 (n = 20)	STAD + RT	≤ 30	2-6	T3-4	≤ 7.1	3.34	1.12 to 9.97	10.9
6 (n = 8)	STAD + RT	≤ 30	2-6	T2c	> 7.1	4.43	1.50 to 13.12	14.2
7 (n = 11)	STAD + RT	> 30	2-6	T3-4	≤ 7.1	4.20	1.16 to 15.24	13.5
8 (n = 23)	STAD + RT	≤ 30	7-10	T3-4	≤ 7.1	4.45	1.19 to 16.70	14.2
9 (n = 6)	STAD + RT	> 30	2-6	T2c	> 7.1	5.57	1.55 to 20.07	17.5
10 (n = 21)	STAD + RT	≤ 30	7-10	T2c	> 7.1	5.91	1.64 to 21.25	18.4
11 (n = 11)	STAD + RT	≤ 30	2-6	T3-4	> 7.1	7.98	2.05 to 31.10	24.0
12 (n = 16)	STAD + RT	> 30	7-10	T3-4	≤ 7.1	5.60	1.35 to 23.28	17.5
13 (n = 11)	STAD + RT	> 30	7-10	T2c	> 7.1	7.42	1.85 to 29.69	22.6
14 (n = 4)	STAD + RT	> 30	2-6	T3-4	> 7.1	10.02	2.19 to 45.86	29.2
15 (n = 25)	STAD + RT	≤ 30	7-10	T3-4	> 7.1	10.63	2.42 to 46.80	30.7
16 (n = 17)	STAD + RT	> 30	7-10	T3-4	> 7.1	13.36	2.77 to 64.42	36.9

Abbreviations: iPSA, initial prostate-specific antigen; SI, staining index; RR, relative risk; DM, distant metastasis; LTAD, long-term androgen deprivation; RT, radiotherapy; STAD, short-term androgen deprivation.
*An interval that does not contain 1 indicates a statistically significant difference between the subgroups at the 0.003 level.
†5-year distant metastasis rates are estimated from Cox proportional hazard model.

dence of distant metastasis when patients with the more favorable features are treated with STAD + RT or LTAD + RT.

DISCUSSION

The immunohistochemical determination of the proportion of cells staining positive for the nuclear expression of the proliferation antigen Ki-67 has long been recognized as a correlate of growth fraction.[1-4] For patients with prostate cancer, the vast majority of papers have shown that Ki-67 staining of prostate tumor tissue, usually from prostatectomy specimens is predictive of patient outcome. Of the relatively few reports of Ki-67 staining of pretreatment diagnostic tissue from patients treated definitively for local-regional prostate cancer with radiotherapy, all have shown that this measure of growth fraction is related to patient outcome.[5-7,14] However, the prior studies have involved small numbers of patients, and there is no clear definition of a cut point that could be applied clinically on a routine basis.

The analysis described here is one of the largest multi-institutional prostate cancer biomarker studies reported. The Ki67-SI, when used as a continuous variable, was found to be the strongest predictor of distant metastasis, cause-specific death and overall death. The higher the Ki67-SI, the greater the risk of an adverse event. The strongest association was observed between the Ki67-SI and distant metastasis.

In order to illustrate the impact of Ki67-SI in univariate analysis and to define a Ki67-SI cut point with clinical applicability, the 3.5%[6,7,16] and 7.1%[16] cut points were tested. These cut points have been observed previously to correlate with patient outcome, including biochemical failure and distant metastasis. The search for a consistent end point has been problematic. The 3.5% cut point was confirmed in two different populations; one consisting of relatively favorable patients treated with radiation alone at M.D. Anderson Cancer Center[6] and the other consisting of locally advanced patients enrolled in RTOG protocol 86-10.[16] In the current study of patients from RTOG protocol 92-02, the 3.5% cut point was not associated with patient outcome. However, the 7.1% Ki67-SI cut point, which was derived from a prior analysis of RTOG protocol 86-10,[16] was strongly predictive of distant metastasis and cause-specific death in the RTOG 92-02 cohort. An investigation of different cut points in RTOG 92-02 revealed a relative risk of distant metastasis of 3.5 to 3.8 with a Ki67-SI of 9 to 10 (Table 6), suggesting that 7.1% is not the optimal cut point in this population. Thus, the most appropriate cut point is still clouded by some inconsistency between databases, which is probably related in part to the prognostic composition and distribution of Ki67-SI values of the patients analyzed. The distribution of Ki67-SI values might be skewed in studies with small patient numbers. The current analysis of about 500 cases with well-defined, mostly high risk features, is by far the most important to date. Although a Ki67-SI of 9 to 10 appears to be optimal in the RTOG 92-02 cohort as a predictor of the DM, the substantiation of the Ki67-SI 7.1% cut point, supports the use of this cut point for the present.

The accurate determination of prognosis before treatment is of the utmost import for patients with prostate cancer. Treatment options are numerous and appropriate selection is based on risk stratification. This is particularly true when the recommendation is for LTAD + RT, because of the pronounced side effects and diminution of quality of life[22,23] from LTAD. In addition to the side effects attributable to LTAD as a single agent, there is also evidence emerging that there is an increase in radiotherapy-related rectal toxicity when androgen deprivation is combined with RT.[24,25] The ability to accurately segregate patients into risk groups has been greatly enhanced by the inclusion of iPSA into models of patient outcome, along with the classical factors of Gleason score and T stage. The promise of biomarkers is that risk stratification will become even more precise and possibly reveal mechanistic relationships in the process. The addition of the Ki67-SI to iPSA, Gleason score, and T stage identified subgroups of patients treated with STAD + RT who had the same DM rate as those with the most favorable prognostic attributes who were treated with LTAD + RT (Table 8); there was no apparent advantage to the use of LTAD in these subgroups. These findings should be considered hypothesis-generating since the numbers of patients in the individual subgroups was small (broad CIs), and it is possible that unknown prognostic factors, aside from iPSA, Gleason score, and T stage, were unevenly distributed.

In summary, the analysis of the Ki67-SI in pretreatment diagnostic prostate biopsy samples from RTOG protocol 92-02 is one of the largest biomarker studies of prostate cancer patients enrolled onto a multi-institutional trial. The results demonstrate a very strong relationship between the Ki67-SI and DM, CSD and OD, which was more significant than the conventional prognostic factors of iPSA, Gleason score and T stage. Although the data presented suggest that a Ki67-SI > 7.1% is associated with a high risk of DM, the optimal cut point has not been firmly established.

Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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MDM2 As A Predictor of Prostate Cancer Outcome: An Analysis of RTOG 8610

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Abstract

Purpose: The MDM2 oncoprotein promotes p53 degradation via ubiquitin, establishing negative feedback control of p53 and consequently affecting cell-cycle arrest and apoptosis. We examined the association between MDM2 expression and local failure (LF), distant metastasis (DM), cause-specific mortality (CSM) and overall mortality (OM) in men treated in RTOG 86-10 with radiotherapy (RT), with or without androgen deprivation.

Experimental Design: Of the 456 eligible and analyzable cases (parent cohort), archival diagnostic tissue from 108 patients had adequate tumor for MDM2 analysis (MDM2 cohort). Cox proportional hazards multivariate analysis (MVA) was used to determine the relationship of MDM2 to the end points. MDM2 overexpression was manually classified as >5% nuclear staining. An image-analysis system (ACIS, Chromavision Medical Systems, Inc., San Juan Capistrano, CA) was also used to quantify the proportion of tumor nuclei with MDM2 staining (ACIS index) and staining intensity.

Results: Overexpression of MDM2 by manual counts was seen in 44% (n=47). In the manual count analysis, there was no significant relationship between MDM2 overexpression and outcome. The ACIS index, using a cutpoint defined by the median value, ≤3% versus >3%, was related to 5-year DM rates in univariate (32.6% versus 45.8%; p=0.057) and multivariate (p=0.06) analyses. The intensity of MDM2 staining was not significant.

Conclusions: MDM2 expression quantified by image-analysis was weakly associated with DM. The cohort examined was relatively small and with larger patient numbers, MDM2 overexpression may emerge as a more significant covariate.

INTRODUCTION

The MDM2 oncoprotein, a ubiquitin ligase, binds to several apoptotic proteins including E2F-1, pRb and p53, but is principally a negative regulator of p53. It is induced by p53, binds to its amino terminal transactivation domain, and consequently inhibits transcription of genes responsible for cell cycle arrest and apoptosis [1-3]. MDM2 oncogene overexpression has been seen in a wide variety of tumors [4], including prostatic carcinomas [5-7], where it has been observed in more than 30% of men. It is associated with high-risk local-regional disease [8] and hormone refractory disease [9]. In our experience, MDM2 suppression via antisense oligonucleotides sensitizes prostate tumor cells in vitro to radiation (RT) [10] and androgen deprivation (AD) [11]. Thus, MDM2 is a promising therapeutic target and the level of expression may be a useful marker of treatment outcome. This is the first study, to our knowledge, examining the predictive value of MDM2 overexpression in men with prostate cancer treated with RT.

Radiation Therapy Oncology Group (RTOG) protocol 8610 was a phase III randomized clinical trial designed to compare the effect of RT plus short-term neoadjuvant and concurrent AD to RT alone [12]. The patients enrolled had locally advanced disease, with palpable tumors of surface area 25 cm^2 or greater. Nearly one-third of the patients had Gleason score 8 to 10 disease and there was documented lymph node involvement in 8%. The purpose of this analysis was to identify the relationship of MDM2 expression to local failure (LF), distant metastasis (DM), cause-specific mortality (CSM) and overall mortality (OM).

MATERIALS and METHODS

Patients Characteristics

RTOG protocol 86-10 has previously been described in detail [12]. The pretreatment diagnostic samples were sectioned and reviewed by the study pathologist (D.J. Grignon). Of the 108 patient samples available for MDM2 analysis, the distribution of patients by Gleason score was 27 with Gleason Score 2-6 and 80 with Gleason Score 7-10 (one patient was missing a Gleason score). The distribution of patients by clinical T-category was 29 with T2 and 79 with T3 disease. Sixty-two and 46 patients were assigned to RT alone and RT+STAD, respectively.

Immunohistochemical Analysis

Sections best representing the tumor were cut 4 μm thick onto poly-L-lysine slides from paraffin-embedded formalin-fixed tissues. The tissue was then deparaffinized in xylene and rehydrated in a series of ethanol washes (95%) to a final distilled water step. Slides were then pressure-cooked in an antigen retrieval citrate buffer solution (pH 6.0) for 50 minutes. After rinsing with water, the slides were covered with 3% hydrogen peroxide for 5 minutes at room temperature, then rinsed in Tris Buffer, and humidified. The primary monoclonal MDM2 antibody (clone IF2, Zymed Laboratories Inc.; 1:100 dilution) was then overlaid. The slides were rinsed in Tris Buffer, then incubated with Biotin (Dako LSAB II Kit) for 10 minutes, rinsed again as before, then incubated with Streptavidin for 10 minutes. After rinsing again with Tris buffer, chromagen (DAB, Research Genetics) was applied for 5 minutes. The slides were then counterstained with commercially-prepared hematoxylin (Dako Corporation, Carpinteria, CA) for 5 minutes,

dehydrated and coverslipped. All staining was performed on a Dako Autostainer. Positive controls with prostate carcinoma tissue sections were used for comparison during tissue analysis.

Two investigators (L-Y.K., T.A-S.) reviewed the slides under a light microscope without knowledge of patient outcome. For the manual analysis, >5% dark brown nuclear tumor cell staining was considered positive, indicating overexpression of MDM2. This was considered a reasonable cutpoint to use since prior analyses considered any positive staining [8], >5% staining [13] to ≥20% staining [7].

The percentage of cells with nuclear staining (ACIS index) and the intensity of staining were also quantified using an image-analysis system (ACIS, Chromavision Medical Systems, Inc., San Juan Capistrano, CA). A color threshold for brown (positive nuclei) and blue (negative nuclei) staining was set for every slide analyzed. Where possible, at least 3 areas of interest in the tissue visualized at X40 magnification were designated for quantification. A final sample mean percent index (ACIS index) was derived by the computer software. Intensity of staining was scored on a grayscale of 0 - 255, where 255 represented black.

The analysis of p53 by immunohistochemistry has been described previously in this patient population [14]. The staining methods used were similar. p53 was deemed positive when > 20% of the tumor cells had nuclear staining, as quantified manually.

Definition of End Points

The four end points examined were LF, DM, CSM and OM. The details of these endpoints have been described previously [12, 14, 15]. Time to failure or death was measured from the date of randomization to the first reported date of failure.

Statistical Analysis

There were 456 assessable patients in the parent cohort of RTOG 86-10 [12, 16]. The MDM2 study cohort was comprised of 108 patients analyzed both manually and by ACIS. As of June 30, 2000, the median follow-up of all surviving patients in the study cohort was 9.3 years and median follow-up of all entered patients was 6.7 years. The distributions of patient characteristics and treatment assignments were compared by the Pearson x^2 test and the Yates correction factor. Estimates of OM were derived using the Kaplan-Meier method [17], whilst the cumulative incidence approach was used to estimate LF, DM and CSM. Multivariate analysis (MVA) using Cox proportional hazard models were applied to each of the end points to identify the impact of MDM2.

There were 348 patients in the parent cohort in whom MDM2 was not quantified. Using the chi-square test, statistical comparisons were carried out to assess if the distributions of patients by prognostic factors were different between the groups.

The MDM2 ACIS index and ACIS intensity score were modeled as continuous and categorical (using a cutpoint at the median value) variables in Cox proportional hazards models.

The interaction between MDM2 and p53 also led us to include the p53 data described in a previous study on RTOG 86-10 [14]. In that study, a cohort of 129 patients

were analyzed for p53-positivity (overexpression) by immunohistochemistry; p53 overexpression was associated with an increase in the incidence of DM.

RESULTS

We determined MDM2 overexpression in 108 (23.7%) of the 456 eligible and analyzable cases in RTOG protocol 86-10. Table 1 shows the distribution of cases in which MDM2 was (MDM2 cohort) and was not (other assessable patients in RTOG-86-10) determined, according to pretreatment characteristics and assigned treatment. There were no statistically significant differences in the distribution of patients by potential prognostic factors between these two groups. Table 2 displays the distribution of patients in the MDM2 cohort by MDM2 manual count results (5% cutpoint) and patient characteristics. The only significant finding was that MDM2 overexpression was significantly associated with higher Gleason scores; 40 (85%) patients with MDM2 overexpression had a Gleason score of 7-10, whereas 7 (15%) had a Gleason score 2-6 ($p=0.029$). MDM2 overexpression was not associated with age, clinical stage, assigned treatment or p53 status.

The univariate analysis results for the MDM2 cohort are shown in Table 3. Although there was no significant relationship between the MDM2 manual count results and outcome, MDM2 overexpression was associated with a 5-year DM rate in univariate analysis of 42.6% versus 28.6% when MDM2 was not overexpressed ($p=0.15$). This observation may be clinically meaningful, given the relatively small number of patients. The analyses, with respect to DM and the other end points tested, may not have been adequately powered to detect a difference in MDM2 expression. For the end point of DM, the power to detect the risk observed in the univariate analysis (relative risk 1.49) was 31%. In the MVA (Table 4), controlling for Gleason score, p53 status and assigned

treatment, the association of the MDM2 manual count results with DM was slightly weaker (p=0.17).

The MDM2 manual count results were obtained using a >5% cutpoint for overexpression. A range of cutpoints have been used in the past [7, 8, 13]. The rationale for using this particular cutpoint was that it is clearly above background and has been used before [13]. However, the results were not statistically significant and there is the possibility that it is not the optimal cutpoint. Hence, we proceeded to use an image-analysis system in order to more precisely quantify the proportion of tumor cells with nuclear MDM2 staining (ACIS index). A median ACIS index of 3.0% (range of 0 – 26.0%) was obtained. Table 5 shows the relationship of the ACIS index to the manual results. When we compared the 75% Quartile cutpoint of 5% to the equivalent manual results, there were some discrepancies: 3 cases, scored 'negative' in manual analysis due to extremes in staining intensity, were scored >5% by ACIS. This is likely due to the ability of ACIS to more accurately score a wide range of staining intensities. Also, 15 of the 24 cases scored positive manually, were scored 5% exactly by ACIS.

The three cutpoints were then applied in univariate analysis to the four end points. A relationship was seen between the median 3% ACIS index cutpoint and DM, as shown in Table 6. MDM2 overexpression in ≤3% of tumor cells was associated with a 5-yr DM rate of 32.6% versus 45.8% when >3% had overexpression (p=0.057) (Figure. 2). A similar level of significance was seen in the MVA (RR=1.85, p=0.06) (Table 7). p53-positivity and Gleason Score 7-10 were significantly associated with DM (RR=2.68, p=0.02; RR= 2.7, p=0.03). When the MDM2 ACIS index was used as a continuous variable in MVA, no relationship to DM or the other end points was observed in MVA.

Finally, MVAs for the MDM2 ACIS intensity score, modeled both as a continuous variable and by the median cutpoint (162 relative units) suggested a relationship with DM when used as a continuous variable (Continuous: p=0.10; Cutpoint: p=0.97). The ACIS intensity score was not found to be associated with any other end point.

DISCUSSION

MDM2 is a key regulator of apoptosis through its interactions with p53, E2F1, pRB and other proteins [18, 19]. We described recently that the apoptotic response of prostate cancer cells to androgen deprivation and/or radiation was significantly affected by the level of MDM2 expression [10, 11] in prostate cancer cell lines. Antisense MDM2 is available as a potential therapeutic adjunct to androgen deprivation and radiation therapy. We investigated the expression of MDM2 in men treated with radiation therapy (RT), with and without short term androgen deprivation (STAD), here to determine if MDM2 overexpression is predictive of patient outcome, as a prelude to targeting men with antisense MDM2 in future trials.

Little is known about the abnormal expression of MDM2 in prostate cancer, as it relates to other prognostic variables and patient outcome. Osman et al [7] found MDM2 overexpression in 33% of 86 radical prostatectomy cases. MDM2 expression was not related to p53 expression, but was associated with advanced stage; no relationship was observed between MDM2 expression and biochemical failure. Leite et al [8] found that MDM2 was overexpressed in over 40% of 118 men who underwent radical prostatectomy and such overexpression was associated strongly with increased tumor volume ($p=0.001$) and weakly with a higher proliferation index ($p=0.046$) and higher tumor stage ($p=0.054$). MDM2 was not associated with p53. In our study, which is the first to investigate such relationships in men treated with RT±STAD, nuclear MDM2 overexpression was observed in 44%. MDM2 overexpression also was not related to p53 expression; but, was related to a higher Gleason score and weakly to distant metastasis.

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The lack of a relationship between p53 and MDM2 expression is possibly due to a lack of feedback from mutant p53.

Our analysis of MDM2 expression was performed in two phases. First, we performed manual counts, assigning an incidence of >5% tumor nuclear positivity to represent overexpression. Of the three prior reports of men with prostate cancer, the categorization of positive overexpression ranged from any nuclear staining to >5% to ≥20% [7, 8, 13]. A value of >5% seemed reasonable, as this could be easily recognized as being above background. However, the cutpoint of 5% is somewhat arbitrary.

The determination of the relationship of the percentage tumor cells demonstrating MDM2 staining was quantified more precisely using an image-analysis system. The resulting ACIS index, while correlating with the manual count results, was more strongly related to the outcome measure of DM. The median ACIS index was 3% and this was chosen as the cutpoint. The ACIS index at the median cutpoint was related to DM in both univariate and multivariate analyses; although statistical significance at the $p<0.05$ level was not obtained ($p=0.06$). Likewise, the ACIS staining intensity was also related to DM, albeit more weakly. The results are promising in that such associations were seen even with relatively small patient numbers and p53 included in the analysis.

In summary, the relationship of MDM2 overexpression to DM in men treated with RT ± AD may be clinically meaningful and should be further investigated in a larger cohort. The predictive value of MDM2 should also be investigated in a more contemporary group of men treated in the PSA era.

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Table 1. Distribution of all patients by presence or absence of MDM2 data (N=456)

Characteristics		Presence (n=108)	Absence (n=348)	p-value*
GLSC	2-6	27(25%)	102(32%)	0.21
	7-10	80(75%)	220(68%)	
	Unknown	1(<1%)	26(7%)	
T-Stage	T2	29(27%)	108(31%)	0.41
	T3	79(73%)	240(69%)	
Assigned Treatment	RT Alone	62(57%)	168(48%)	0.10
	RT + STAD	46(43%)	180(52%)	
p53	Negative	70(86%)	36(75%)	0.10
	Positive	11(14%)	12(25%)	
	Unknown	27	300	

*Chi-square statistics

Abbreviations: GLSC, Gleason Score; RT, radiation therapy;

STAD, short term androgen deprivation.

Table 2. Distribution of patients by MDM2 manual count results

Characteristics		Negative (n=61)	Positive (n=47)	p-value*
Age	<75	46(75%)	35(74%)	0.91
	≥75	15(25%)	12(26%)	
GLSC	2-6	20(33%)	7(15%)	0.029
	7-10	40(67%)	40(85%)	
T-Stage	T2	17(28%)	12(26%)	0.79
	T3	44(72%)	35(74%)	
Assigned Treatment	RT Alone	33(54%)	29(62%)	0.43
	RT+STAD	28(46%)	18(38%)	
p53	Negative	34(83%)	36(90%)	0.35
	Positive	7(17%)	4(10%)	
	Unknown	20	7	

*Chi-square statistics

[+]One patient is missing Gleason score in the "Negative" MDM2 group.

Abbreviations: GLSC, Gleason Score; RT, radiation therapy;

STAD, short term androgen deprivation.

Table 3. Univariate analysis results for the MDM2 cohort (N=108)

	Local Failure	Distant Metastasis	Cause Specific Mortality	Overall Mortality
MDM2	0.92 (0.49, 1.76) p=0.81	1.49 (0.87, 2.56) p=0.15	1.32 (0.70, 2.49) p=0.40	1.12 (0.71, 1.74) p=0.63

The relative risk and 95% confidence intervals (in parenthesis) are shown;

P-values were derived from the Chi-square test.

Table 4. Multivariate analysis of distant metastasis; MDM2 manual count results

Variable[#]	Group	RR[*]	p-value[†]
MDM2	Positive	1.60(0.82, 3.10)	0.17
GLSC	7-10	2.66(1.07, 6.63)	0.0353
STAD	Yes	0.89(0.47, 1.68)	0.71
p53	Positive	2.67(1.17, 6.10)	0.0199

[#]All variables were dichotomous.

[*]Relative Risk: a risk ratio of 1 indicates no difference between the two subgroups.

[†]P-value from Chi-square test using the Cox proportional hazards model.

Abbreviations: GLSC, Gleason Score; STAD, short term androgen deprivation.

Table 5. Distribution of manual vs. ACIS Index results

ACIS	Manual		Total	p-value*
	Negative (n=61)	Positive (n=47)		
≤1.0	28(46%)	0	28	<0.0001
>1.0	33(54%)	47(100%)	80	
≤3.0	54(89%)	5 (11%)	59	<0.0001
>3.0	7(11%)	42(89%)	49	
≤5.0	58(95%)	24(51%)	82	<0.0001
>5.0	3 (5%)	23(49%)	26	

*Chi-square statistics

Table 6. Univariate analysis of the MDM2 ACIS Index

Endpoint	MDM2 ACIS Cutpoint	n	Failures	RR*	(95% CI)	p-value†	5-Year Rate	5-Year (95% CI)
LF	≤3.0	59	21	1.07	(0.57,2.03)	0.83	25.4%	(14.2%, 36.7%)
	>3.0	49	18				23.0%	(10.9%, 35.7%)
DM	≤3.0	59	26	1.69	(0.98,2.91)	0.057	32.6%	(20.4%, 44.8%)
	>3.0	49	28				45.8%	(31.4%, 60.1%)
CSM	≤3.0	59	19	1.29	(0.68,2.44)	0.43	18.6%	(8.6%, 28.7%)
	>3.0	49	19				27.1%	(14.4%, 39.9%)
OM	≤3.0	59	42	1.13	(0.72,1.77)	0.59	33.9%	(21.6%,46.2%)
	>3.0	49	36				41.6%	(27.4%, 55.8%)

*Relative Risk: a risk ratio of 1 indicates no difference between the two subgroups.

The MDM2 ACIS index indicator was coded as 0: ≤*cutpoint*, 1: >*cutpoint*.

†P-value from Chi-square test using the Cox proportional hazards model.

Abbreviations: LF, local failure; DM, distant metastasis;

CSM, cause specific mortality; OM, overall mortality.

Table 7. Multivariate analysis of distant metastasis with MDM2 ACIS Index

Variable[#]	Group	RR[*]	p-value[†]
MDM2	>3.0	1.85(0.97, 3.56)	0.06
GLSC	7-10	2.70(1.09, 6.71)	0.0328
STAD	Yes	0.89(0.47, 1.68)	0.72
p53	Positive	2.68(1.18, 6.07)	0.0181

[#]All variables were dichotomous.

[*]Relative Risk: a risk ratio of 1 indicates no difference between the two subgroups.

[†]P-value from Chi-square test using the Cox proportional hazards model.

Abbreviations: GLSC, Gleason Score; STAD, short term androgen deprivation.

Figure 1

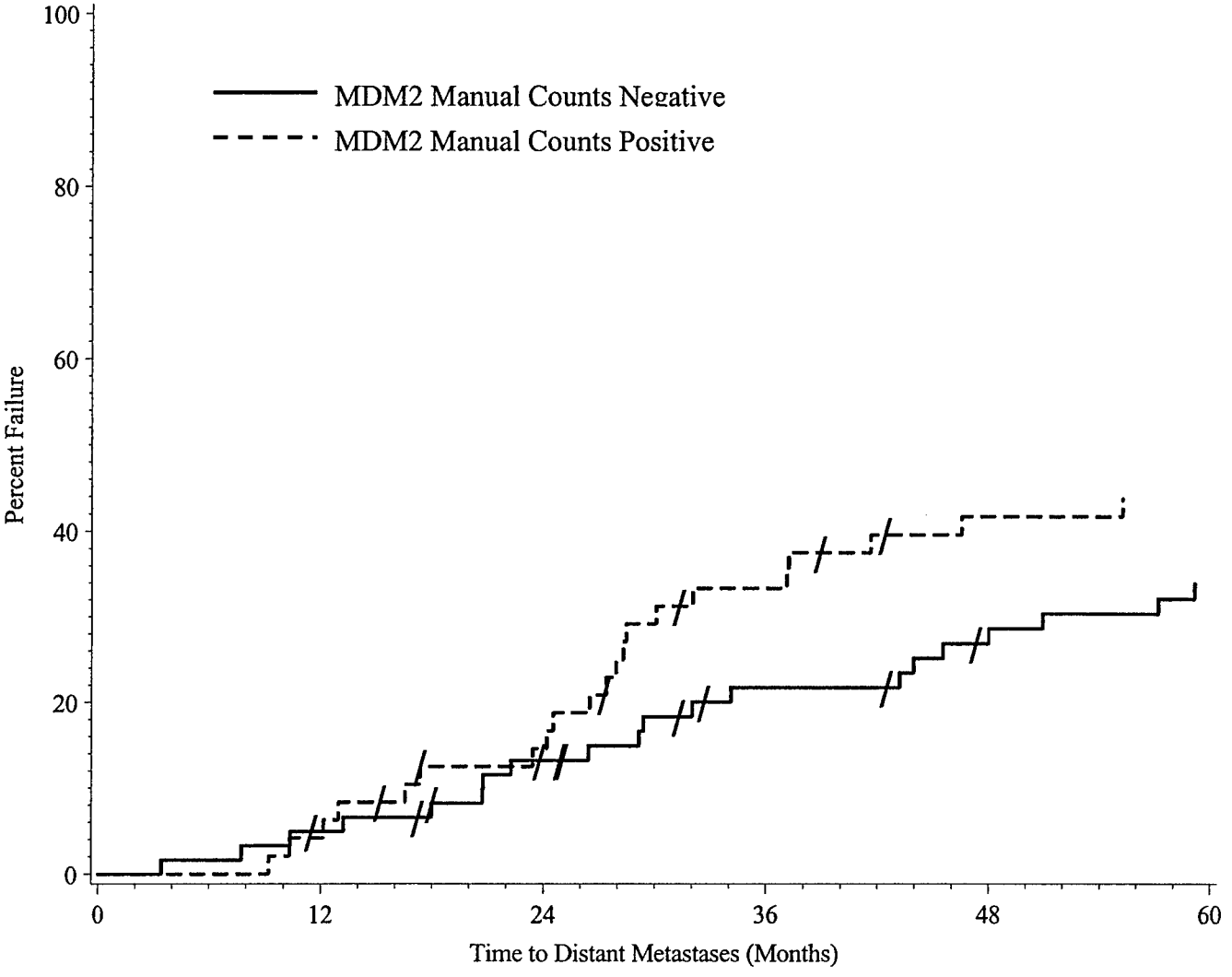
MDM2 Manual Counts Negative
MDM2 Manual Counts Positive
Percent Failure
100
80
60
40
20
0
0
12
24
36
48
60
Time to Distant Metastases (Months)

Figure 2

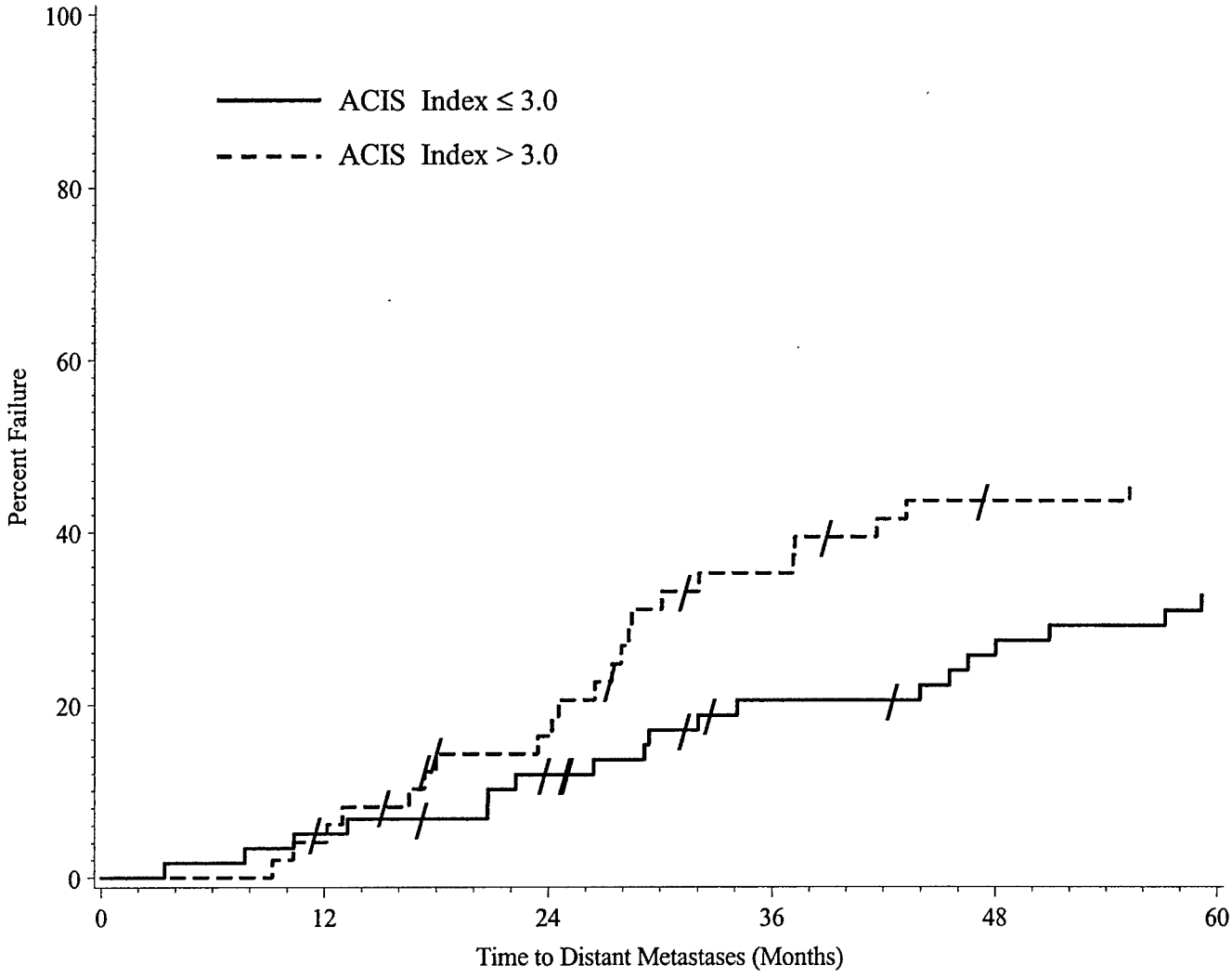
ACIS Index ≤ 3.0
ACIS Index > 3.0
Percent Failure
100
80
60
40
20
0
0
12
24
36
48
60
Time to Distant Metastases (Months)

Figure Legends

Figure 1. Survival Curve of DM by MDM2 manual count results, using cumulative incidence estimates.

Figure 2. Survival Curve of DM by MDM2 ACIS Index at the 3% cutpoint, using cumulative incidence estimates.